culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10.) After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 ul of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4 degree C.

[1333] To verify the expression of the polypeptide, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus containing the polynucleotide at a multiplicity of infection ("MOI") of about 2. If radiolabeled proteins are desired, 6 hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). After 42 hours, 5 uCi of ^{35}S-methionine and 5 uCi ^{35}S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled).

[1334] Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the produced protein.

[1335] Example 8: Expression of a Polypeptide in Mammalian Cells

[1336] The polypeptide of the present invention can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

[1337] Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), pCMVSport 2.0, and pCMVSport 3.0. Mammalian host cells that could be used include, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

[1338] Alternatively, the polypeptide can be expressed in stable cell lines containing the polynucleotide integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

[1339] The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of interest. (See, e.g., Alt, F. W., et al., J. Biol. Chem. 253:1357-1370 (1978); Hamlin, J. L. and Ma, C., Biochem. et Biophys. Acta, 1097:107-143 (1990); Page, M. J. and Sydenham, M. A., Biotechnology 9:64-68 (1991).) Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., Biochem J. 227:277-279 (1991); Bebbington et al., Bio/Technology 10:169-175 (1992). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

[1340] Derivatives of the plasmid pSV2-dhfr (ATCC Accession No. 37146), the expression vectors pC4 (ATCC Accession No. 209646) and pC6 (ATCC Accession No.209647) contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., Cell 41:521-530 (1985).) Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors also contain the 3' intron, the

polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter.

[1341] Specifically, the plasmid pC6, for example, is digested with appropriate restriction enzymes and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

[1342] A polynucleotide of the present invention is amplified according to the protocol outlined in Example 1. If the naturally occurring signal sequence is used to produce the secreted protein, the vector does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891.)

[1343] The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

[1344] The amplified fragment is then digested with the same restriction enzyme and purified on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 using, for instance, restriction enzyme analysis.

[1345] Chinese hamster ovary cells lacking an active DHFR gene is used for transfection. Five μg of the expression plasmid pC6 a pC4 is cotransfected with 0.5 ug of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of metothrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing

even higher concentrations of methotrexate (1 uM, 2 uM, 5 uM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 uM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

[1346] Example 9: Protein Fusions

[1347] The polypeptides of the present invention are preferably fused to other proteins. These fusion proteins can be used for a variety of applications. For example, fusion of the present polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See Example 5; see also EP A 394,827; Traunecker, et al., Nature 331:84-86 (1988).) Similarly, fusion to IgG-1, IgG-3, and albumin increases the halflife time in vivo. Nuclear localization signals fused to the polypeptides of the present invention can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule, or the protocol described in Example 5.

[1348] Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector.

[1349] For example, if pC4 (Accession No. 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and a polynucleotide of the present invention, isolated by the PCR protocol described in Example 1, is ligated into this BamHI site. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

[1350] If the naturally occurring signal sequence is used to produce the secreted protein, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891.)

[1351] Human IgG Fc region:
GGGATCCGGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGC
CCAGCACCTGAATTCGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCCAAAA
CCCAAGGACACCCTCATGATCTCCCGGACTCCTGAGGTCACATGCGTGGT
GGTGGACGTAAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGG
ACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTA
CAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACT
GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCA
ACCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAAC
CACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAG
GTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAAGCGACATCGCCGT
GGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCT
CCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTG
GACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCA
TGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG
GTAAATGAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO:1)

[1352] **Example 10: Production of an Antibody from a Polypeptide**

[1353] The antibodies of the present invention can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, cells expressing a polypeptide of the present invention is administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of the secreted protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

[1354] In the most preferred method, the antibodies of the present invention are monoclonal antibodies (or protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology. (Köhler et al., Nature 256:495 (1975); Köhler et al., Eur. J. Immunol. 6:511 (1976); Köhler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981).) In general, such procedures involve immunizing an animal (preferably a mouse) with polypeptide or, more preferably, with a secreted polypeptide-expressing cell. Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56 degrees C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 ug/ml of streptomycin.

[1355] The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981).) The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the polypeptide.

[1356] Alternatively, additional antibodies capable of binding to the polypeptide can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the protein-specific antibody can be blocked by the polypeptide. Such antibodies comprise anti-idiotypic antibodies to the protein-specific antibody and can be used to immunize an animal to induce formation of further protein-specific antibodies.

[1357] It will be appreciated that Fab and F(ab')2 and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). Alternatively, secreted protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry.

[1358] For in vivo use of antibodies in humans, it may be preferable to use "humanized" chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985).)

[1359] Example 11: Production Of Secreted Protein For High-Throughput Screening Assays

[1360] The following protocol produces a supernatant containing a polypeptide to be tested. This supernatant can then be used in the Screening Assays described herein.

[1361] First, dilute Poly-D-Lysine (644 587 Boehringer-Mannheim) stock solution (1mg/ml in PBS) 1:20 in PBS (w/o calcium or magnesium 17-516F Biowhittaker) for a working solution of 50ug/ml. Add 200 ul of this solution to each well (24 well plates) and incubate at RT for 20 minutes. Be sure to distribute the solution over each well (note: a 12-channel pipetter may be used with tips on every other channel). Aspirate off the Poly-D-Lysine solution and rinse with 1ml PBS (Phosphate Buffered Saline). The PBS should remain in the well until just prior to plating the cells and plates may be poly-lysine coated in advance for up to two weeks.

[1362] Plate 293T cells (do not carry cells past P+20) at 2 x 10^5 cells/well in .5ml DMEM(Dulbecco's Modified Eagle Medium)(with 4.5 G/L glucose and L-glutamine

(12-604F Biowhittaker))/10% heat inactivated FBS(14-503F Biowhittaker)/1x Penstrep(17-602E Biowhittaker). Let the cells grow overnight.

[1363] The next day, mix together in a sterile solution basin: 300 ul Lipofectamine (18324-012 Gibco/BRL) and 5ml Optimem I (31985070 Gibco/BRL)/96-well plate. With a small volume multi-channel pipetter, aliquot approximately 2ug of an expression vector containing a polynucleotide insert, produced by the methods described in Examples 8 or 9, into an appropriately labeled 96-well round bottom plate. With a multi-channel pipetter, add 50ul of the Lipofectamine/Optimem I mixture to each well. Pipette up and down gently to mix. Incubate at RT 15-45 minutes. After about 20 minutes, use a multi-channel pipetter to add 150ul Optimem I to each well. As a control, one plate of vector DNA lacking an insert should be transfected with each set of transfections.

[1364] Preferably, the transfection should be performed by tag-teaming the following tasks. By tag-teaming, hands on time is cut in half, and the cells do not spend too much time on PBS. First, person A aspirates off the media from four 24-well plates of cells, and then person B rinses each well with .5-1ml PBS. Person A then aspirates off PBS rinse, and person B, using a12-channel pipetter with tips on every other channel, adds the 200ul of DNA/Lipofectamine/Optimem I complex to the odd wells first, then to the even wells, to each row on the 24-well plates. Incubate at 37 degrees C for 6 hours.

[1365] While cells are incubating, prepare appropriate media, either 1%BSA in DMEM with 1x penstrep, or CHO-5 media (116.6 mg/L of CaCl2 (anhyd); 0.00130 mg/L $CuSO_4$-$5H_2O$; 0.050 mg/L of $Fe(NO_3)_3$-$9H_2O$; 0.417 mg/L of $FeSO_4$-$7H_2O$; 311.80 mg/L of Kcl; 28.64 mg/L of $MgCl_2$; 48.84 mg/L of $MgSO_4$; 6995.50 mg/L of NaCl; 2400.0 mg/L of $NaHCO_3$; 62.50 mg/L of NaH_2PO_4-H_20; 71.02 mg/L of Na_2HPO4; .4320 mg/L of $ZnSO_4$-$7H_2O$; .002 mg/L of Arachidonic Acid ; 1.022 mg/L of Cholesterol; .070 mg/L of DL-alpha-Tocopherol-Acetate; 0.0520 mg/L of Linoleic Acid; 0.010 mg/L of Linolenic Acid; 0.010 mg/L of Myristic Acid; 0.010 mg/L of Oleic Acid; 0.010 mg/L of Palmitric Acid; 0.010 mg/L of Palmitic Acid; 100 mg/L of Pluronic F-68; 0.010 mg/L of Stearic Acid; 2.20 mg/L of Tween 80; 4551 mg/L of D-Glucose; 130.85 mg/ml of L- Alanine; 147.50 mg/ml of L-Arginine-HCL; 7.50 mg/ml

of L-Asparagine-H_20; 6.65 mg/ml of L-Aspartic Acid; 29.56 mg/ml of L-Cystine-2HCL-H_20; 31.29 mg/ml of L-Cystine-2HCL; 7.35 mg/ml of L-Glutamic Acid; 365.0 mg/ml of L-Glutamine; 18.75 mg/ml of Glycine; 52.48 mg/ml of L-Histidine-HCL-H_20; 106.97 mg/ml of L-Isoleucine; 111.45 mg/ml of L-Leucine; 163.75 mg/ml of L-Lysine HCL; 32.34 mg/ml of L-Methionine; 68.48 mg/ml of L-Phenylalainine; 40.0 mg/ml of L-Proline; 26.25 mg/ml of L-Serine; 101.05 mg/ml of L-Threonine; 19.22 mg/ml of L-Tryptophan; 91.79 mg/ml of L-Tryrosine-2Na-2H_20; 99.65 mg/ml of L-Valine; 0.0035 mg/L of Biotin; 3.24 mg/L of D-Ca Pantothenate; 11.78 mg/L of Choline Chloride; 4.65 mg/L of Folic Acid; 15.60 mg/L of i-Inositol; 3.02 mg/L of Niacinamide; 3.00 mg/L of Pyridoxal HCL; 0.031 mg/L of Pyridoxine HCL; 0.319 mg/L of Riboflavin; 3.17 mg/L of Thiamine HCL; 0.365 mg/L of Thymidine; and 0.680 mg/L of Vitamin B_{12}; 25 mM of HEPES Buffer; 2.39 mg/L of Na Hypoxanthine; 0.105 mg/L of Lipoic Acid; 0.081 mg/L of Sodium Putrescine-2HCL; 55.0 mg/L of Sodium Pyruvate; 0.0067 mg/L of Sodium Selenite; 20uM of Ethanolamine; 0.122 mg/L of Ferric Citrate; 41.70 mg/L of Methyl-B-Cyclodextrin complexed with Linoleic Acid; 33.33 mg/L of Methyl-B-Cyclodextrin complexed with Oleic Acid; and 10 mg/L of Methyl-B-Cyclodextrin complexed with Retinal) with 2mm glutamine and 1x penstrep. (BSA (81-068-3 Bayer) 100gm dissolved in 1L DMEM for a 10% BSA stock solution). Filter the media and collect 50 ul for endotoxin assay in 15ml polystyrene conical.

[1366] The transfection reaction is terminated, preferably by tag-teaming, at the end of the incubation period. Person A aspirates off the transfection media, while person B adds 1.5ml appropriate media to each well. Incubate at 37 degrees C for 45 or 72 hours depending on the media used: 1%BSA for 45 hours or CHO-5 for 72 hours.

[1367] On day four, using a 300ul multichannel pipetter, aliquot 600ul in one 1ml deep well plate and the remaining supernatant into a 2ml deep well. The supernatants from each well can then be used in the assays described in Examples 13-20.

[1368] It is specifically understood that when activity is obtained in any of the assays described below using a supernatant, the activity originates from either the polypeptide directly (e.g., as a secreted protein) or by the polypeptide inducing expression of other proteins, which are then secreted into the supernatant. Thus, the

invention further provides a method of identifying the protein in the supernatant characterized by an activity in a particular assay.

[1369] Example 12: Construction of GAS Reporter Construct

[1370] One signal transduction pathway involved in the differentiation and proliferation of cells is called the Jaks-STATs pathway. Activated proteins in the Jaks-STATs pathway bind to gamma activation site "GAS" elements or interferon-sensitive responsive element ("ISRE"), located in the promoter of many genes. The binding of a protein to these elements alter the expression of the associated gene.

[1371] GAS and ISRE elements are recognized by a class of transcription factors called Signal Transducers and Activators of Transcription, or "STATs." There are six members of the STATs family. Stat1 and Stat3 are present in many cell types, as is Stat2 (as response to IFN-alpha is widespread). Stat4 is more restricted and is not in many cell types though it has been found in T helper class I, cells after treatment with IL-12. Stat5 was originally called mammary growth factor, but has been found at higher concentrations in other cells including myeloid cells. It can be activated in tissue culture cells by many cytokines.

[1372] The STATs are activated to translocate from the cytoplasm to the nucleus upon tyrosine phosphorylation by a set of kinases known as the Janus Kinase ("Jaks") family. Jaks represent a distinct family of soluble tyrosine kinases and include Tyk2, Jak1, Jak2, and Jak3. These kinases display significant sequence similarity and are generally catalytically inactive in resting cells.

[1373] The Jaks are activated by a wide range of receptors summarized in the Table below. (Adapted from review by Schidler and Darnell, Ann. Rev. Biochem. 64:621-51 (1995).) A cytokine receptor family, capable of activating Jaks, is divided into two groups: (a) Class 1 includes receptors for IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15, Epo, PRL, GH, G-CSF, GM-CSF, LIF, CNTF, and thrombopoietin; and (b) Class 2 includes IFN-a, IFN-g, and IL-10. The Class 1 receptors share a conserved cysteine motif (a set of four conserved cysteines and one tryptophan) and a WSXWS motif (a membrane proximal region encoding Trp-Ser-Xxx-Trp-Ser (SEQ ID NO:2)).

[1374] Thus, on binding of a ligand to a receptor, Jaks are activated, which in turn activate STATs, which then translocate and bind to GAS elements. This entire process is encompassed in the Jaks-STATs signal transduction pathway.

[1375] Therefore, activation of the Jaks-STATs pathway, reflected by the binding of the GAS or the ISRE element, can be used to indicate proteins involved in the proliferation and differentiation of cells. For example, growth factors and cytokines are known to activate the Jaks-STATs pathway. (See Table below.) Thus, by using GAS elements linked to reporter molecules, activators of the Jaks-STATs pathway can be identified.

Ligand	JAKs tyk2	Jak1	Jak2	Jak3	STATS	GAS(elements) or ISRE
IFN family						
IFN-a/B	+	+	-	-	1,2,3	ISRE
IFN-g		+	+	-	1	GAS (IRF1>Lys6>IFP)
Il-10	+	?	?	-	1,3	
gp130 family						
IL-6 (Pleiotrophic)	+	+	+	?	1,3	GAS (IRF1>Lys6>IFP)
Il-11(Pleiotrophic)	?	+	?	?	1,3	
OnM(Pleiotrophic)	?	+	+	?	1,3	
LIF(Pleiotrophic)	?	+	+	?	1,3	
CNTF(Pleiotrophic)	-/+	+	+	?	1,3	
G-CSF(Pleiotrophic)	?	+	?	?	1,3	
IL-12(Pleiotrophic)	+	-	+	+	1,3	
g-C family						
IL-2 (lymphocytes)	-	+	-	+	1,3,5	GAS
IL-4 (lymph/myeloid)	-	+	-	+	6	GAS (IRF1 = IFP >>Ly6)(IgH)
IL-7 (lymphocytes)	-	+	-	+	5	GAS
IL-9 (lymphocytes)	-	+	-	+	5	GAS
IL-13 (lymphocyte)	-	+	?	?	6	GAS
IL-15	?	+	?	+	5	GAS
gp140 family						
IL-3 (myeloid)	-	-	+	-	5	GAS (IRF1>IFP>>Ly6)
IL-5 (myeloid)	-	-	+	-	5	GAS
GM-CSF (myeloid)	-	-	+	-	5	GAS
Growth hormone family						
GH	?	-	+	-	5	
PRL	?	+/-	+	-	1,3,5	
EPO	?	-	+	-	5	GAS(B-CAS>IRF1=IFP>>Ly6)
Receptor Tyrosine Kinases						
EGF	?	+	+	-	1,3	GAS (IRF1)
PDGF	?	+	+	-	1,3	
CSF-1	?	+	+	-	1,3	GAS (not IRF1)

[1376] To construct a synthetic GAS containing promoter element, which is used in the Biological Assays described in Examples 13-14, a PCR based strategy is employed to generate a GAS-SV40 promoter sequence. The 5' primer contains four tandem copies of the GAS binding site found in the IRF1 promoter and previously demonstrated to bind STATs upon induction with a range of cytokines (Rothman et al., Immunity 1:457-468 (1994).), although other GAS or ISRE elements can be used instead. The 5' primer also contains 18bp of sequence complementary to the SV40 early promoter sequence and is flanked with an XhoI site. The sequence of the 5' primer is:

5':GCGCCTCGAGATTTCCCCGAAATCTAGATTTCCCCGAAATGATTTCCCC GAAATGATTTCCCCGAAATATCTGCCATCTCAATTAG:3' (SEQ ID NO:3)

[1377] The downstream primer is complementary to the SV40 promoter and is flanked with a Hind III site: 5':GCGGCAAGCTTTTTGCAAAGCCTAGGC:3' (SEQ ID NO:4).

[1378] PCR amplification is performed using the SV40 promoter template present in the B-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI/Hind III and subcloned into BLSK2-. (Stratagene.) Sequencing with forward and reverse primers confirms that the insert contains the following sequence:

5':CTCGAGATTTCCCCGAAATCTAGATTTCCCCGAAATGATTTCCCCGAAA TGATTTCCCCGAAATATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCG CCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCT CCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCC TCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCT AGGCTTTTGCAAAAAGCTT:3' (SEQ ID NO:5).

[1379] With this GAS promoter element linked to the SV40 promoter, a GAS:SEAP2 reporter construct is next engineered. Here, the reporter molecule is a secreted alkaline phosphatase, or "SEAP." Clearly, however, any reporter molecule can be instead of SEAP, in this or in any of the other Examples. Well known reporter molecules that can be used instead of SEAP include chloramphenicol

acetyltransferase (CAT), luciferase, alkaline phosphatase, B-galactosidase, green fluorescent protein (GFP), or any protein detectable by an antibody.

[1380] The above sequence confirmed synthetic GAS-SV40 promoter element is subcloned into the pSEAP-Promoter vector obtained from Clontech using HindIII and XhoI, effectively replacing the SV40 promoter with the amplified GAS:SV40 promoter element, to create the GAS-SEAP vector. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

[1381] Thus, in order to generate mammalian stable cell lines expressing the GAS-SEAP reporter, the GAS-SEAP cassette is removed from the GAS-SEAP vector using SalI and NotI, and inserted into a backbone vector containing the neomycin resistance gene, such as pGFP-1 (Clontech), using these restriction sites in the multiple cloning site, to create the GAS-SEAP/Neo vector. Once this vector is transfected into mammalian cells, this vector can then be used as a reporter molecule for GAS binding as described in Examples 13-14.

[1382] Other constructs can be made using the above description and replacing GAS with a different promoter sequence. For example, construction of reporter molecules containing NFK-B and EGR promoter sequences are described in Examples 15 and 16. However, many other promoters can be substituted using the protocols described in these Examples. For instance, SRE, IL-2, NFAT, or Osteocalcin promoters can be substituted, alone or in combination (e.g., GAS/NF-KB/EGR, GAS/NF-KB, Il-2/NFAT, or NF-KB/GAS). Similarly, other cell lines can be used to test reporter construct activity, such as HELA (epithelial), HUVEC (endothelial), Reh (B-cell), Saos-2 (osteoblast), HUVAC (aortic), or Cardiomyocyte.

[1383] Example 13: High-Throughput Screening Assay for T-cell Activity.

[1384] The following protocol is used to assess T-cell activity by identifying factors, and determining whether supernate containing a polypeptide of the invention proliferates and/or differentiates T-cells. T-cell activity is assessed using the GAS/SEAP/Neo construct produced in Example 12. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway.

The T-cell used in this assay is Jurkat T-cells (ATCC Accession No. TIB-152), although Molt-3 cells (ATCC Accession No. CRL-1552) and Molt-4 cells (ATCC Accession No. CRL-1582) cells can also be used.

[1385] Jurkat T-cells are lymphoblastic CD4+ Th1 helper cells. In order to generate stable cell lines, approximately 2 million Jurkat cells are transfected with the GAS-SEAP/neo vector using DMRIE-C (Life Technologies)(transfection procedure described below). The transfected cells are seeded to a density of approximately 20,000 cells per well and transfectants resistant to 1 mg/ml genticin selected. Resistant colonies are expanded and then tested for their response to increasing concentrations of interferon gamma. The dose response of a selected clone is demonstrated.

[1386] Specifically, the following protocol will yield sufficient cells for 75 wells containing 200 ul of cells. Thus, it is either scaled up, or performed in multiple to generate sufficient cells for multiple 96 well plates. Jurkat cells are maintained in RPMI + 10% serum with 1%Pen-Strep. Combine 2.5 mls of OPTI-MEM (Life Technologies) with 10 ug of plasmid DNA in a T25 flask. Add 2.5 ml OPTI-MEM containing 50 ul of DMRIE-C and incubate at room temperature for 15-45 mins.

[1387] During the incubation period, count cell concentration, spin down the required number of cells (10^7 per transfection), and resuspend in OPTI-MEM to a final concentration of 10^7 cells/ml. Then add 1ml of 1 x 10^7 cells in OPTI-MEM to T25 flask and incubate at 37 degrees C for 6 hrs. After the incubation, add 10 ml of RPMI + 15% serum.

[1388] The Jurkat:GAS-SEAP stable reporter lines are maintained in RPMI + 10% serum, 1 mg/ml Genticin, and 1% Pen-Strep. These cells are treated with supernatants containing polypeptides of the invention and/or induced polypeptides of the invention as produced by the protocol described in Example 11.

[1389] On the day of treatment with the supernatant, the cells should be washed and resuspended in fresh RPMI + 10% serum to a density of 500,000 cells per ml. The exact number of cells required will depend on the number of supernatants being screened. For one 96 well plate, approximately 10 million cells (for 10 plates, 100 million cells) are required.

[1390] Transfer the cells to a triangular reservoir boat, in order to dispense the cells into a 96 well dish, using a 12 channel pipette. Using a 12 channel pipette, transfer 200 ul of cells into each well (therefore adding 100, 000 cells per well).

[1391] After all the plates have been seeded, 50 ul of the supernatants are transferred directly from the 96 well plate containing the supernatants into each well using a 12 channel pipette. In addition, a dose of exogenous interferon gamma (0.1, 1.0, 10 ng) is added to wells H9, H10, and H11 to serve as additional positive controls for the assay.

[1392] The 96 well dishes containing Jurkat cells treated with supernatants are placed in an incubator for 48 hrs (note: this time is variable between 48-72 hrs). 35 ul samples from each well are then transferred to an opaque 96 well plate using a 12 channel pipette. The opaque plates should be covered (using sellophene covers) and stored at –20 degrees C until SEAP assays are performed according to Example 17. The plates containing the remaining treated cells are placed at 4 degrees C and serve as a source of material for repeating the assay on a specific well if desired.

[1393] As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate Jurkat T cells. Over 30 fold induction is typically observed in the positive control wells.

[1394] The above protocol may be used in the generation of both transient, as well as, stable transfected cells, which would be apparent to those of skill in the art.

[1395] Example 14: High-Throughput Screening Assay Identifying Myeloid Activity

[1396] The following protocol is used to assess myeloid activity by determining whether polypeptides of the invention proliferates and/or differentiates myeloid cells. Myeloid cell activity is assessed using the GAS/SEAP/Neo construct produced in Example 12. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The myeloid cell used in this assay is U937, a pre-monocyte cell line, although TF-1, HL60, or KG1 can be used.

[1397] To transiently transfect U937 cells with the GAS/SEAP/Neo construct produced in Example 12, a DEAE-Dextran method (Kharbanda et. al., 1994, Cell

Growth & Differentiation, 5:259-265) is used. First, harvest $2x10e^7$ U937 cells and wash with PBS. The U937 cells are usually grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

[1398] Next, suspend the cells in 1 ml of 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 mg/ml DEAE-Dextran, 8 ug GAS-SEAP2 plasmid DNA, 140 mM NaCl, 5 mM KCl, 375 uM $Na_2HPO_4.7H_2O$, 1 mM $MgCl_2$, and 675 uM $CaCl_2$. Incubate at 37 degrees C for 45 min.

[1399] Wash the cells with RPMI 1640 medium containing 10% FBS and then resuspend in 10 ml complete medium and incubate at 37 degrees C for 36 hr.

[1400] The GAS-SEAP/U937 stable cells are obtained by growing the cells in 400 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 400 ug/ml G418 for couple of passages.

[1401] These cells are tested by harvesting $1x10^8$ cells (this is enough for ten 96-well plates assay) and wash with PBS. Suspend the cells in 200 ml above described growth medium, with a final density of $5x10^5$ cells/ml. Plate 200 ul cells per well in the 96-well plate (or $1x10^5$ cells/well).

[1402] Add 50 ul of the supernatant prepared by the protocol described in Example 11. Incubate at 37 degrees C for 48 to 72 hr. As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate U937 cells. Over 30 fold induction is typically observed in the positive control wells. SEAP assay the supernatant according to the protocol described in Example 17.

[1403] Example 15: High-Throughput Screening Assay Identifying Neuronal Activity.

[1404] When cells undergo differentiation and proliferation, a group of genes are activated through many different signal transduction pathways. One of these genes, EGR1 (early growth response gene 1), is induced in various tissues and cell types upon activation. The promoter of EGR1 is responsible for such induction. Using the EGR1 promoter linked to reporter molecules, activation of cells can be assessed.

[1405] Particularly, the following protocol is used to assess neuronal activity in PC12 cell lines. PC12 cells (rat phenochromocytoma cells) are known to proliferate and/or differentiate by activation with a number of mitogens, such as TPA (tetradecanoyl phorbol acetate), NGF (nerve growth factor), and EGF (epidermal growth factor). The EGR1 gene expression is activated during this treatment. Thus, by stably transfecting PC12 cells with a construct containing an EGR promoter linked to SEAP reporter, activation of PC12 cells can be assessed.

[1406] The EGR/SEAP reporter construct can be assembled by the following protocol. The EGR-1 promoter sequence (-633 to +1)(Sakamoto K et al., Oncogene 6:867-871 (1991)) can be PCR amplified from human genomic DNA using the following primers:

5' GCGCTCGAGGGATGACAGCGATAGAACCCCGG -3' (SEQ ID NO:6)

5' GCGAAGCTTCGCGACTCCCCGGATCCGCCTC-3' (SEQ ID NO:7).

[1407] Using the GAS:SEAP/Neo vector produced in Example 12, EGR1 amplified product can then be inserted into this vector. Linearize the GAS:SEAP/Neo vector using restriction enzymes XhoI/HindIII, removing the GAS/SV40 stuffer. Restrict the EGR1 amplified product with these same enzymes. Ligate the vector and the EGR1 promoter.

[1408] To prepare 96 well-plates for cell culture, two mls of a coating solution (1:30 dilution of collagen type I (Upstate Biotech Inc. Cat#08-115) in 30% ethanol (filter sterilized)) is added per one 10 cm plate or 50 ml per well of the 96-well plate, and allowed to air dry for 2 hr.

[1409] PC12 cells are routinely grown in RPMI-1640 medium (Bio Whittaker) containing 10% horse serum (JRH BIOSCIENCES, Cat. # 12449-78P), 5% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 ug/ml streptomycin on a precoated 10 cm tissue culture dish. One to four split is done every three to four days. Cells are removed from the plates by scraping and resuspended with pipetting up and down for more than 15 times.

[1410] Transfect the EGR/SEAP/Neo construct into PC12 using the Lipofectamine protocol described in Example 11. EGR-SEAP/PC12 stable cells are obtained by growing the cells in 300 ug/ml G418. The G418-free medium is used for routine

growth but every one to two months, the cells should be re-grown in 300 ug/ml G418 for couple of passages.

[1411] To assay for neuronal activity, a 10 cm plate with cells around 70 to 80% confluent is screened by removing the old medium. Wash the cells once with PBS (Phosphate buffered saline). Then starve the cells in low serum medium (RPMI-1640 containing 1% horse serum and 0.5% FBS with antibiotics) overnight.

[1412] The next morning, remove the medium and wash the cells with PBS. Scrape off the cells from the plate, suspend the cells well in 2 ml low serum medium. Count the cell number and add more low serum medium to reach final cell density as $5x10^5$ cells/ml.

[1413] Add 200 ul of the cell suspension to each well of 96-well plate (equivalent to $1x10^5$ cells/well). Add 50 ul supernatant produced by Example 11, 37°C for 48 to 72 hr. As a positive control, a growth factor known to activate PC12 cells through EGR can be used, such as 50 ng/ul of Neuronal Growth Factor (NGF). Over fifty-fold induction of SEAP is typically seen in the positive control wells. SEAP assay the supernatant according to Example 17.

[1414] **Example 16: High-Throughput Screening Assay for T-cell Activity**

[1415] NF-KB (Nuclear Factor KB) is a transcription factor activated by a wide variety of agents including the inflammatory cytokines IL-1 and TNF, CD30 and CD40, lymphotoxin-alpha and lymphotoxin-beta, by exposure to LPS or thrombin, and by expression of certain viral gene products. As a transcription factor, NF-KB regulates the expression of genes involved in immune cell activation, control of apoptosis (NF- KB appears to shield cells from apoptosis), B and T-cell development, anti-viral and antimicrobial responses, and multiple stress responses.

[1416] In non-stimulated conditions, NF- KB is retained in the cytoplasm with I-KB (Inhibitor KB). However, upon stimulation, I- KB is phosphorylated and degraded, causing NF- KB to shuttle to the nucleus, thereby activating transcription of target genes. Target genes activated by NF- KB include IL-2, IL-6, GM-CSF, ICAM-1 and class 1 MHC.

[1417] Due to its central role and ability to respond to a range of stimuli, reporter constructs utilizing the NF-KB promoter element are used to screen the supernatants produced in Example 11. Activators or inhibitors of NF-KB would be useful in treating diseases. For example, inhibitors of NF-KB could be used to treat those diseases related to the acute or chronic activation of NF-KB, such as rheumatoid arthritis.

[1418] To construct a vector containing the NF-KB promoter element, a PCR based strategy is employed. The upstream primer contains four tandem copies of the NF-KB binding site (GGGGACTTTCCC) (SEQ ID NO:8), 18 bp of sequence complementary to the 5' end of the SV40 early promoter sequence, and is flanked with an XhoI site:

5':GCGGCCTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGAC TTTCCATCCTGCCATCTCAATTAG:3' (SEQ ID NO:9)

[1419] The downstream primer is complementary to the 3' end of the SV40 promoter and is flanked with a Hind III site:

5':GCGGCAAGCTTTTTGCAAAGCCTAGGC:3' (SEQ ID NO:4)

[1420] PCR amplification is performed using the SV40 promoter template present in the pB-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI and Hind III and subcloned into BLSK2-. (Stratagene) Sequencing with the T7 and T3 primers confirms the insert contains the following sequence:

5':CTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGACTTTCC ATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCC ATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGA CTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTA TTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAA GCTT:3' (SEQ ID NO:10)

[1421] Next, replace the SV40 minimal promoter element present in the pSEAP2-promoter plasmid (Clontech) with this NF-KB/SV40 fragment using XhoI and

HindIII. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

[1422] In order to generate stable mammalian cell lines, the NF-KB/SV40/SEAP cassette is removed from the above NF-KB/SEAP vector using restriction enzymes SalI and NotI, and inserted into a vector containing neomycin resistance. Particularly, the NF-KB/SV40/SEAP cassette was inserted into pGFP-1 (Clontech), replacing the GFP gene, after restricting pGFP-1 with SalI and NotI.

[1423] Once NF-KB/SV40/SEAP/Neo vector is created, stable Jurkat T-cells are created and maintained according to the protocol described in Example 13. Similarly, the method for assaying supernatants with these stable Jurkat T-cells is also described in Example 13. As a positive control, exogenous TNF alpha (0.1,1, 10 ng) is added to wells H9, H10, and H11, with a 5-10 fold activation typically observed.

[1424] Example 17: Assay for SEAP Activity

[1425] As a reporter molecule for the assays described in Examples 13-16, SEAP activity is assayed using the Tropix Phospho-light Kit (Cat. BP-400) according to the following general procedure. The Tropix Phospho-light Kit supplies the Dilution, Assay, and Reaction Buffers used below.

[1426] Prime a dispenser with the 2.5x Dilution Buffer and dispense 15 ul of 2.5x dilution buffer into Optiplates containing 35 ul of a supernatant. Seal the plates with a plastic sealer and incubate at 65 degree C for 30 min. Separate the Optiplates to avoid uneven heating.

[1427] Cool the samples to room temperature for 15 minutes. Empty the dispenser and prime with the Assay Buffer. Add 50 ml Assay Buffer and incubate at room temperature 5 min. Empty the dispenser and prime with the Reaction Buffer (see the table below). Add 50 ul Reaction Buffer and incubate at room temperature for 20 minutes. Since the intensity of the chemiluminescent signal is time dependent, and it takes about 10 minutes to read 5 plates on luminometer, one should treat 5 plates at each time and start the second set 10 minutes later.

[1428] Read the relative light unit in the luminometer. Set H12 as blank, and print the results. An increase in chemiluminescence indicates reporter activity.

Reaction Buffer Formulation:

# of plates	Rxn buffer diluent (ml)	CSPD (ml)
10	60	3
11	65	3.25
12	70	3.5
13	75	3.75
14	80	4
15	85	4.25
16	90	4.5
17	95	4.75
18	100	5
19	105	5.25
20	110	5.5
21	115	5.75
22	120	6
23	125	6.25
24	130	6.5
25	135	6.75
26	140	7
27	145	7.25
28	150	7.5
29	155	7.75
30	160	8
31	165	8.25
32	170	8.5
33	175	8.75
34	180	9
35	185	9.25
36	190	9.5
37	195	9.75
38	200	10
39	205	10.25
40	210	10.5
41	215	10.75
42	220	11
43	225	11.25
44	230	11.5
45	235	11.75
46	240	12
47	245	12.25
48	250	12.5
49	255	12.75
50	260	13

[1429] Example 18: High-Throughput Screening Assay Identifying Changes in Small Molecule Concentration and Membrane Permeability

[1430] Binding of a ligand to a receptor is known to alter intracellular levels of small molecules, such as calcium, potassium, sodium, and pH, as well as alter membrane potential. These alterations can be measured in an assay to identify supernatants

which bind to receptors of a particular cell. Although the following protocol describes an assay for calcium, this protocol can easily be modified to detect changes in potassium, sodium, pH, membrane potential, or any other small molecule which is detectable by a fluorescent probe.

[1431] The following assay uses Fluorometric Imaging Plate Reader ("FLIPR") to measure changes in fluorescent molecules (Molecular Probes) that bind small molecules. Clearly, any fluorescent molecule detecting a small molecule can be used instead of the calcium fluorescent molecule, fluo-4 (Molecular Probes, Inc.; catalog no. F-14202), used here.

[1432] For adherent cells, seed the cells at 10,000 -20,000 cells/well in a Co-star black 96-well plate with clear bottom. The plate is incubated in a CO_2 incubator for 20 hours. The adherent cells are washed two times in Biotek washer with 200 ul of HBSS (Hank's Balanced Salt Solution) leaving 100 ul of buffer after the final wash.

[1433] A stock solution of 1 mg/ml fluo-4 is made in 10% pluronic acid DMSO. To load the cells with fluo-4 , 50 ul of 12 ug/ml fluo-4 is added to each well. The plate is incubated at 37 degrees C in a CO_2 incubator for 60 min. The plate is washed four times in the Biotek washer with HBSS leaving 100 ul of buffer.

[1434] For non-adherent cells, the cells are spun down from culture media. Cells are re-suspended to $2\text{-}5x10^6$ cells/ml with HBSS in a 50-ml conical tube. 4 ul of 1 mg/ml fluo-4 solution in 10% pluronic acid DMSO is added to each ml of cell suspension. The tube is then placed in a 37 degrees C water bath for 30-60 min. The cells are washed twice with HBSS, resuspended to $1x10^6$ cells/ml, and dispensed into a microplate, 100 ul/well. The plate is centrifuged at 1000 rpm for 5 min. The plate is then washed once in Denley CellWash with 200 ul, followed by an aspiration step to 100 ul final volume.

[1435] For a non-cell based assay, each well contains a fluorescent molecule, such as fluo-4 . The supernatant is added to the well, and a change in fluorescence is detected.

[1436] To measure the fluorescence of intracellular calcium, the FLIPR is set for the following parameters: (1) System gain is 300-800 mW; (2) Exposure time is 0.4 second; (3) Camera F/stop is F/2; (4) Excitation is 488 nm; (5) Emission is 530 nm;

and (6) Sample addition is 50 ul. Increased emission at 530 nm indicates an extracellular signaling event which has resulted in an increase in the intracellular Ca^{++} concentration.

[1437] Example 19: High-Throughput Screening Assay Identifying Tyrosine Kinase Activity

[1438] The Protein Tyrosine Kinases (PTK) represent a diverse group of transmembrane and cytoplasmic kinases. Within the Receptor Protein Tyrosine Kinase RPTK) group are receptors for a range of mitogenic and metabolic growth factors including the PDGF, FGF, EGF, NGF, HGF and Insulin receptor subfamilies. In addition there are a large family of RPTKs for which the corresponding ligand is unknown. Ligands for RPTKs include mainly secreted small proteins, but also membrane-bound and extracellular matrix proteins.

[1439] Activation of RPTK by ligands involves ligand-mediated receptor dimerization, resulting in transphosphorylation of the receptor subunits and activation of the cytoplasmic tyrosine kinases. The cytoplasmic tyrosine kinases include receptor associated tyrosine kinases of the src-family (e.g., src, yes, lck, lyn, fyn) and non-receptor linked and cytosolic protein tyrosine kinases, such as the Jak family, members of which mediate signal transduction triggered by the cytokine superfamily of receptors (e.g., the Interleukins, Interferons, GM-CSF, and Leptin).

[1440] Because of the wide range of known factors capable of stimulating tyrosine kinase activity, the identification of novel human secreted proteins capable of activating tyrosine kinase signal transduction pathways are of interest. Therefore, the following protocol is designed to identify those novel human secreted proteins capable of activating the tyrosine kinase signal transduction pathways.

[1441] Seed target cells (e.g., primary keratinocytes) at a density of approximately 25,000 cells per well in a 96 well Loprodyne Silent Screen Plates purchased from Nalge Nunc (Naperville, IL). The plates are sterilized with two 30 minute rinses with 100% ethanol, rinsed with water and dried overnight. Some plates are coated for 2 hr with 100 ml of cell culture grade type I collagen (50 mg/ml), gelatin (2%) or polylysine (50 mg/ml), all of which can be purchased from Sigma Chemicals (St.

Louis, MO) or 10% Matrigel purchased from Becton Dickinson (Bedford,MA), or calf serum, rinsed with PBS and stored at 4 degree C. Cell growth on these plates is assayed by seeding 5,000 cells/well in growth medium and indirect quantitation of cell number through use of alamarBlue as described by the manufacturer Alamar Biosciences, Inc. (Sacramento, CA) after 48 hr. Falcon plate covers #3071 from Becton Dickinson (Bedford,MA) are used to cover the Loprodyne Silent Screen Plates. Falcon Microtest III cell culture plates can also be used in some proliferation experiments.

[1442] To prepare extracts, A431 cells are seeded onto the nylon membranes of Loprodyne plates (20,000/200ml/well) and cultured overnight in complete medium. Cells are quiesced by incubation in serum-free basal medium for 24 hr. After 5-20 minutes treatment with EGF (60ng/ml) or 50 ul of the supernatant produced in Example 11, the medium was removed and 100 ml of extraction buffer ((20 mM HEPES pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 2 mM Na3VO4, 2 mM Na4P2O7 and a cocktail of protease inhibitors (# 1836170) obtained from Boeheringer Mannheim (Indianapolis, IN) is added to each well and the plate is shaken on a rotating shaker for 5 minutes at 4 degrees C. The plate is then placed in a vacuum transfer manifold and the extract filtered through the 0.45 mm membrane bottoms of each well using house vacuum. Extracts are collected in a 96-well catch/assay plate in the bottom of the vacuum manifold and immediately placed on ice. To obtain extracts clarified by centrifugation, the content of each well, after detergent solubilization for 5 minutes, is removed and centrifuged for 15 minutes at 4 degrees C at 16,000 x g.

[1443] Test the filtered extracts for levels of tyrosine kinase activity. Although many methods of detecting tyrosine kinase activity are known, one method is described here.

[1444] Generally, the tyrosine kinase activity of a supernatant is evaluated by determining its ability to phosphorylate a tyrosine residue on a specific substrate (a biotinylated peptide). Biotinylated peptides that can be used for this purpose include PSK1 (corresponding to amino acids 6-20 of the cell division kinase cdc2-p34) and

PSK2 (corresponding to amino acids 1-17 of gastrin). Both peptides are substrates for a range of tyrosine kinases and are available from Boehringer Mannheim.

[1445] The tyrosine kinase reaction is set up by adding the following components in order. First, add 10ul of 5uM Biotinylated Peptide, then 10ul ATP/Mg_{2+} (5mM ATP/50mM $MgCl_2$), then 10ul of 5x Assay Buffer (40mM imidazole hydrochloride, pH7.3, 40 mM beta-glycerophosphate, 1mM EGTA, 100mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mg/ml BSA), then 5ul of Sodium Vanadate(1mM), and then 5ul of water. Mix the components gently and preincubate the reaction mix at 30 degrees C for 2 min. Initial the reaction by adding 10ul of the control enzyme or the filtered supernatant.

[1446] The tyrosine kinase assay reaction is then terminated by adding 10 ul of 120mm EDTA and place the reactions on ice.

[1447] Tyrosine kinase activity is determined by transferring 50 ul aliquot of reaction mixture to a microtiter plate (MTP) module and incubating at 37 degrees C for 20 min. This allows the streptavadin coated 96 well plate to associate with the biotinylated peptide. Wash the MTP module with 300ul/well of PBS four times. Next add 75 ul of anti-phospotyrosine antibody conjugated to horse radish peroxidase(anti-P-Tyr-POD(0.5u/ml)) to each well and incubate at 37 degrees C for one hour. Wash the well as above.

[1448] Next add 100ul of peroxidase substrate solution (Boehringer Mannheim) and incubate at room temperature for at least 5 mins (up to 30 min). Measure the absorbance of the sample at 405 nm by using ELISA reader. The level of bound peroxidase activity is quantitated using an ELISA reader and reflects the level of tyrosine kinase activity.

[1449] Example 20: High-Throughput Screening Assay Identifying Phosphorylation Activity

[1450] As a potential alternative and/or compliment to the assay of protein tyrosine kinase activity described in Example 19, an assay which detects activation (phosphorylation) of major intracellular signal transduction intermediates can also be used. For example, as described below one particular assay can detect tyrosine phosphorylation of the Erk-1 and Erk-2 kinases. However, phosphorylation of other

molecules, such as Raf, JNK, p38 MAP, Map kinase kinase (MEK), MEK kinase, Src, Muscle specific kinase (MuSK), IRAK, Tec, and Janus, as well as any other phosphoserine, phosphotyrosine, or phosphothreonine molecule, can be detected by substituting these molecules for Erk-1 or Erk-2 in the following assay.

[1451] Specifically, assay plates are made by coating the wells of a 96-well ELISA plate with 0.1ml of protein G (1ug/ml) for 2 hr at room temp, (RT). The plates are then rinsed with PBS and blocked with 3% BSA/PBS for 1 hr at RT. The protein G plates are then treated with 2 commercial monoclonal antibodies (100ng/well) against Erk-1and Erk-2 (1 hr at RT) (Santa Cruz Biotechnology). (To detect other molecules, this step can easily be modified by substituting a monoclonal antibody detecting any of the above described molecules.) After 3-5 rinses with PBS, the plates are stored at 4 degrees C until use.

[1452] A431 cells are seeded at 20,000/well in a 96-well Loprodyne filterplate and cultured overnight in growth medium. The cells are then starved for 48 hr in basal medium (DMEM) and then treated with EGF (6ng/well) or 50 ul of the supernatants obtained in Example 11 for 5-20 minutes. The cells are then solubilized and extracts filtered directly into the assay plate.

[1453] After incubation with the extract for 1 hr at RT, the wells are again rinsed. As a positive control, a commercial preparation of MAP kinase (10ng/well) is used in place of A431 extract. Plates are then treated with a commercial polyclonal (rabbit) antibody (1ug/ml) which specifically recognizes the phosphorylated epitope of the Erk-1 and Erk-2 kinases (1 hr at RT). This antibody is biotinylated by standard procedures. The bound polyclonal antibody is then quantitated by successive incubations with Europium-streptavidin and Europium fluorescence enhancing reagent in the Wallac DELFIA instrument (time-resolved fluorescence). An increased fluorescent signal over background indicates a phosphorylation.

[1454] Example 21: Method of Determining Alterations in a Gene Corresponding to a Polynucleotide

[1455] RNA isolated from entire families or individual patients presenting with a phenotype of interest (such as a disease) is be isolated. cDNA is then generated from

these RNA samples using protocols known in the art. (See, Sambrook.) The cDNA is then used as a template for PCR, employing primers surrounding regions of interest in SEQ ID NO:X. Suggested PCR conditions consist of 35 cycles at 95 degrees C for 30 seconds; 60-120 seconds at 52-58 degrees C; and 60-120 seconds at 70 degrees C, using buffer solutions described in Sidransky et al., Science 252:706 (1991).

[1456] PCR products are then sequenced using primers labeled at their 5' end with T4 polynucleotide kinase, employing SequiTherm Polymerase. (Epicentre Technologies). The intron-exon borders of selected exons is also determined and genomic PCR products analyzed to confirm the results. PCR products harboring suspected mutations is then cloned and sequenced to validate the results of the direct sequencing.

[1457] PCR products is cloned into T-tailed vectors as described in Holton et al., Nucleic Acids Research, 19:1156 (1991) and sequenced with T7 polymerase (United States Biochemical). Affected individuals are identified by mutations not present in unaffected individuals.

[1458] Genomic rearrangements are also observed as a method of determining alterations in a gene corresponding to a polynucleotide. Genomic clones isolated according to Example 2 are nick-translated with digoxigenindeoxy-uridine 5'-triphosphate (Boehringer Manheim), and FISH performed as described in Johnson et al., Methods Cell Biol. 35:73-99 (1991). Hybridization with the labeled probe is carried out using a vast excess of human cot-1 DNA for specific hybridization to the corresponding genomic locus.

[1459] Chromosomes are counterstained with 4,6-diamino-2-phenylidole and propidium iodide, producing a combination of C- and R-bands. Aligned images for precise mapping are obtained using a triple-band filter set (Chroma Technology, Brattleboro, VT) in combination with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and variable excitation wavelength filters. (Johnson et al., Genet. Anal. Tech. Appl., 8:75 (1991).) Image collection, analysis and chromosomal fractional length measurements are performed using the ISee Graphical Program System. (Inovision Corporation, Durham, NC.) Chromosome alterations of the genomic region hybridized by the probe are identified as insertions, deletions, and

translocations. These alterations are used as a diagnostic marker for an associated disease.

[1460] Example 22: Method of Detecting Abnormal Levels of a Polypeptide in a Biological Sample

[1461] A polypeptide of the present invention can be detected in a biological sample, and if an increased or decreased level of the polypeptide is detected, this polypeptide is a marker for a particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their particular needs.

[1462] For example, antibody-sandwich ELISAs are used to detect polypeptides in a sample, preferably a biological sample. Wells of a microtiter plate are coated with specific antibodies, at a final concentration of 0.2 to 10 ug/ml. The antibodies are either monoclonal or polyclonal and are produced by the method described in Example 10. The wells are blocked so that non-specific binding of the polypeptide to the well is reduced.

[1463] The coated wells are then incubated for > 2 hours at RT with a sample containing the polypeptide. Preferably, serial dilutions of the sample should be used to validate results. The plates are then washed three times with deionized or distilled water to remove unbounded polypeptide.

[1464] Next, 50 ul of specific antibody-alkaline phosphatase conjugate, at a concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate.

[1465] Add 75 ul of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution to each well and incubate 1 hour at room temperature. Measure the reaction by a microtiter plate reader. Prepare a standard curve, using serial dilutions of a control sample, and plot polypeptide concentration on the X-axis (log scale) and fluorescence or absorbance of the Y-axis (linear scale). Interpolate the concentration of the polypeptide in the sample using the standard curve.

[1466] Example 23: Formulation

[1467] The invention also provides methods of treatment and/or prevention diseases, disorders, and/or conditions (such as, for example, any one or more of the diseases or disorders disclosed herein) by administration to a subject of an effective amount of a Therapeutic. By therapeutic is meant a polynucleotides or polypeptides of the invention (including fragments and variants), agonists or antagonists thereof, and/or antibodies thereto, in combination with a pharmaceutically acceptable carrier type (e.g., a sterile carrier).

[1468] The Therapeutic will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the Therapeutic alone), the site of delivery, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" for purposes herein is thus determined by such considerations.

[1469] As a general proposition, the total pharmaceutically effective amount of the Therapeutic administered parenterally per dose will be in the range of about 1ug/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the Therapeutic is typically administered at a dose rate of about 1 ug/kg/hour to about 50 ug/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

[1470] Therapeutics can be are administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), bucally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any. The term

"parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

[1471] Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics are administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), bucally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

[1472] Therapeutics of the invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release Therapeutics include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, e.g., films, or mirocapsules), suitable hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt).

[1473] Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman et al., Biopolymers 22:547-556 (1983)), poly (2- hydroxyethyl methacrylate) (Langer et al., J. Biomed. Mater. Res. 15:167-277 (1981), and Langer, Chem. Tech. 12:98-105 (1982)), ethylene vinyl acetate (Langer et al., Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).

[1474] Sustained-release Therapeutics also include liposomally entrapped Therapeutics of the invention (*see* generally, Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 317 -327 and 353-365 (1989)). Liposomes containing the Therapeutic are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci.(USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP

88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal Therapeutic.

[1475] In yet an additional embodiment, the Therapeutics of the invention are delivered by way of a pump (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)).

[1476] Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

[1477] For parenteral administration, in one embodiment, the Therapeutic is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to the Therapeutic.

[1478] Generally, the formulations are prepared by contacting the Therapeutic uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

[1479] The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum

albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, manose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

[1480] The Therapeutic is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of polypeptide salts.

[1481] Any pharmaceutical used for therapeutic administration can be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutics generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

[1482] Therapeutics ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous Therapeutic solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized Therapeutic using bacteriostatic Water-for-Injection.

[1483] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the Therapeutics of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the Therapeutics may be employed in conjunction with other therapeutic compounds.

[1484] The Therapeutics of the invention may be administered alone or in combination with adjuvants. Adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, alum, alum plus

deoxycholate (ImmunoAg), MTP-PE (Biocine Corp.), QS21 (Genentech, Inc.), BCG, and MPL. In a specific embodiment, Therapeutics of the invention are administered in combination with alum. In another specific embodiment, Therapeutics of the invention are administered in combination with QS-21. Further adjuvants that may be administered with the Therapeutics of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts, MF-59, and Virosomal adjuvant technology. Vaccines that may be administered with the Therapeutics of the invention include, but are not limited to, vaccines directed toward protection against MMR (measles, mumps, rubella), polio, varicella, tetanus/diptheria, hepatitis A, hepatitis B, haemophilus influenzae B, whooping cough, pneumonia, influenza, Lyme's Disease, rotavirus, cholera, yellow fever, Japanese encephalitis, poliomyelitis, rabies, typhoid fever, and pertussis. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

[1485] The Therapeutics of the invention may be administered alone or in combination with other therapeutic agents. Therapeutic agents that may be administered in combination with the Therapeutics of the invention, include but not limited to, other members of the TNF family, chemotherapeutic agents, antibiotics, steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic agents, cytokines and/or growth factors. Combinations may be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination"

further includes the separate administration of one of the compounds or agents given first, followed by the second.

[1486] In one embodiment, the Therapeutics of the invention are administered in combination with members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the Therapeutics of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma (International Publication No. WO 96/14328), AIM-I (International Publication No. WO 97/33899), endokine-alpha (International Publication No. WO 98/07880), TR6 (International Publication No. WO 98/30694), OPG, and neutrokine-alpha (International Publication No. WO 98/18921, OX40, and nerve growth factor (NGF), and soluble forms of Fas, CD30, CD27, CD40 and 4-IBB, TR2 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), DR4 (International Publication No. WO 98/32856), TR5 (International Publication No. WO 98/30693), TR6 (International Publication No. WO 98/30694), TR7 (International Publication No. WO 98/41629), TRANK, TR9 (International Publication No. WO 98/56892),TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, and soluble forms CD154, CD70, and CD153.

[1487] In certain embodiments, Therapeutics of the invention are administered in combination with antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors. Nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, RETROVIR™ (zidovudine/AZT), VIDEX™ (didanosine/ddI), HIVID™ (zalcitabine/ddC), ZERIT™ (stavudine/d4T), EPIVIR™ (lamivudine/3TC), and COMBIVIR™ (zidovudine/lamivudine). Non-nucleoside reverse transcriptase inhibitors that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, VIRAMUNE™ (nevirapine), RESCRIPTOR™ (delavirdine), and SUSTIVA™ (efavirenz). Protease inhibitors that may be administered in combination

with the Therapeutics of the invention, include, but are not limited to, CRIXIVAN™ (indinavir), NORVIR™ (ritonavir), INVIRASE™ (saquinavir), and VIRACEPT™ (nelfinavir). In a specific embodiment, antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors may be used in any combination with Therapeutics of the invention to treat AIDS and/or to prevent or treat HIV infection.

[1488] In other embodiments, Therapeutics of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the Therapeutics of the invention, include, but are not limited to, TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, ATOVAQUONE™, ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, ETHAMBUTOL™, RIFABUTIN™, CLARITHROMYCIN™, AZITHROMYCIN™, GANCICLOVIR™, FOSCARNET™, CIDOFOVIR™, FLUCONAZOLE™, ITRACONAZOLE™, KETOCONAZOLE™, ACYCLOVIR™, FAMCICOLVIR™, PYRIMETHAMINE™, LEUCOVORIN™, NEUPOGEN™ (filgrastim/G-CSF), and LEUKINE™ (sargramostim/GM-CSF). In a specific embodiment, Therapeutics of the invention are used in any combination with TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, and/or ATOVAQUONE™ to prophylactically treat or prevent an opportunistic *Pneumocystis carinii* pneumonia infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, and/or ETHAMBUTOL™ to prophylactically treat or prevent an opportunistic *Mycobacterium avium* complex infection. In another specific embodiment, Therapeutics of the invention are used in any combination with RIFABUTIN™, CLARITHROMYCIN™, and/or AZITHROMYCIN™ to prophylactically treat or prevent an opportunistic *Mycobacterium tuberculosis* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with GANCICLOVIR™, FOSCARNET™, and/or CIDOFOVIR™ to prophylactically treat or prevent an opportunistic cytomegalovirus infection. In another specific embodiment,

Therapeutics of the invention are used in any combination with FLUCONAZOLE™, ITRACONAZOLE™, and/or KETOCONAZOLE™ to prophylactically treat or prevent an opportunistic fungal infection. In another specific embodiment, Therapeutics of the invention are used in any combination with ACYCLOVIR™ and/or FAMCICOLVIR™ to prophylactically treat or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific embodiment, Therapeutics of the invention are used in any combination with PYRIMETHAMINE™ and/or LEUCOVORIN™ to prophylactically treat or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, Therapeutics of the invention are used in any combination with LEUCOVORIN™ and/or NEUPOGEN™ to prophylactically treat or prevent an opportunistic bacterial infection.

[1489] In a further embodiment, the Therapeutics of the invention are administered in combination with an antiviral agent. Antiviral agents that may be administered with the Therapeutics of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine.

[1490] In a further embodiment, the Therapeutics of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the Therapeutics of the invention include, but are not limited to, amoxicillin, beta-lactamases, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, ciprofloxacin, erythromycin, fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamthoxazole, and vancomycin.

[1491] Conventional nonspecific immunosuppressive agents, that may be administered in combination with the Therapeutics of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T cells.

[1492] In specific embodiments, Therapeutics of the invention are administered in combination with immunosuppressants. Immunosuppressants preparations that may

be administered with the Therapeutics of the invention include, but are not limited to, ORTHOCLONE™ (OKT3), SANDIMMUNE™/NEORAL™/SANGDYA™ (cyclosporin), PROGRAF™ (tacrolimus), CELLCEPT™ (mycophenolate), Azathioprine, glucorticosteroids, and RAPAMUNE™ (sirolimus). In a specific embodiment, immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

[1493] In an additional embodiment, Therapeutics of the invention are administered alone or in combination with one or more intravenous immune globulin preparations. Intravenous immune globulin preparations that may be administered with the Therapeutics of the invention include, but not limited to, GAMMAR™, IVEEGAM™, SANDOGLOBULIN™, GAMMAGARD S/D™, and GAMIMUNE™. In a specific embodiment, Therapeutics of the invention are administered in combination with intravenous immune globulin preparations in transplantation therapy (e.g., bone marrow transplant).

[1494] In an additional embodiment, the Therapeutics of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the Therapeutics of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

[1495] In another embodiment, compostions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the Therapeutics of the invention include, but are not limited to, antibiotic derivatives (e.g., doxorubicin, bleomycin, daunorubicin, and dactinomycin); antiestrogens (e.g., tamoxifen); antimetabolites (e.g., fluorouracil, 5-FU, methotrexate, floxuridine, interferon alpha-2b, glutamic acid, plicamycin,

mercaptopurine, and 6-thioguanine); cytotoxic agents (e.g., carmustine, BCNU, lomustine, CCNU, cytosine arabinoside, cyclophosphamide, estramustine, hydroxyurea, procarbazine, mitomycin, busulfan, cis-platin, and vincristine sulfate); hormones (e.g., medroxyprogesterone, estramustine phosphate sodium, ethinyl estradiol, estradiol, megestrol acetate, methyltestosterone, diethylstilbestrol diphosphate, chlorotrianisene, and testolactone); nitrogen mustard derivatives (e.g., mephalen, chorambucil, mechlorethamine (nitrogen mustard) and thiotepa); steroids and combinations (e.g., bethamethasone sodium phosphate); and others (e.g., dicarbazine, asparaginase, mitotane, vincristine sulfate, vinblastine sulfate, and etoposide).

[1496] In a specific embodiment, Therapeutics of the invention are administered in combination with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or any combination of the components of CHOP. In another embodiment, Therapeutics of the invention are administered in combination with Rituximab. In a further embodiment, Therapeutics of the invention are administered with Rituxmab and CHOP, or Rituxmab and any combination of the components of CHOP.

[1497] In an additional embodiment, the Therapeutics of the invention are administered in combination with cytokines. Cytokines that may be administered with the Therapeutics of the invention include, but are not limited to, IL2, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, IL15, anti-CD40, CD40L, IFN-gamma and TNF-alpha. In another embodiment, Therapeutics of the invention may be administered with any interleukin, including, but not limited to, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, and IL-21.

[1498] In an additional embodiment, the Therapeutics of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the Therapeutics of the invention include, but are not limited to, Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as

disclosed in European Patent Number EP-282317; Placental Growth Factor (PlGF), as disclosed in International Publication Number WO 92/06194; Placental Growth Factor-2 (PlGF-2), as disclosed in Hauser et al., Gorwth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B (VEGF-3); Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832; and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are incorporated herein by reference herein.

[1499] In an additional embodiment, the Therapeutics of the invention are administered in combination with hematopoietic growth factors. Hematopoietic growth factors that may be administered with the Therapeutics of the invention include, but are not limited to, LEUKINE™ (SARGRAMOSTIM™) and NEUPOGEN™ (FILGRASTIM™).

[1500] In an additional embodiment, the Therapeutics of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the Therapeutics of the invention include, but are not limited to, FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

[1501] In additional embodiments, the Therapeutics of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

[1502] Example 24: Method of Treating Decreased Levels of the Polypeptide

[1503] The present invention relates to a method for treating an individual in need of an increased level of a polypeptide of the invention in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of an agonist of the invention (including polypeptides of the invention). Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of a secreted protein in an individual can be treated by administering the polypeptide of the present invention, preferably in the secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of the polypeptide comprising administering to such an individual a Therapeutic comprising an amount of the polypeptide to increase the activity level of the polypeptide in such an individual.

[1504] For example, a patient with decreased levels of a polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in the secreted form. The exact details of the dosing scheme, based on administration and formulation, are provided in Example 23.

[1505] **Example 25: Method of Treating Increased Levels of the Polypeptide**

[1506] The present invention also relates to a method of treating an individual in need of a decreased level of a polypeptide of the invention in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of an antagonist of the invention (including polypeptides and antibodies of the invention).

[1507] In one example, antisense technology is used to inhibit production of a polypeptide of the present invention. This technology is one example of a method of decreasing levels of a polypeptide, preferably a secreted form, due to a variety of etiologies, such as cancer. For example, a patient diagnosed with abnormally increased levels of a polypeptide is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the treatment was well tolerated. The formulation of the antisense polynucleotide is provided in Example 23.

[1508] **Example 26: Method of Treatment Using Gene Therapy-Ex Vivo**

[1509] One method of gene therapy transplants fibroblasts, which are capable of expressing a polypeptide, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

[1510] At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

[1511] pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

[1512] The cDNA encoding a polypeptide of the present invention can be amplified using PCR primers which correspond to the 5' and 3' end sequences respectively as set forth in Example 1 using primers and having appropriate restriction sites and initiation/stop codons, if necessary. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector has the gene of interest properly inserted.

[1513] The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with

10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

[1514] Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether protein is produced.

[1515] The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

[1516] Example 27: Gene Therapy Using Endogenous Genes Corresponding To Polynucleotides of the Invention

[1517] Another method of gene therapy according to the present invention involves operably associating the endogenous polynucleotide sequence of the invention with a promoter via homologous recombination as described, for example, in U.S. Patent NO: 5,641,670, issued June 24, 1997; International Publication NO: WO 96/29411, published September 26, 1996; International Publication NO: WO 94/12650, published August 4, 1994; Koller et al., *Proc. Natl. Acad. Sci. USA,* 86:8932-8935 (1989); and Zijlstra et al., *Nature,* 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

[1518] Polynucleotide constructs are made which contain a promoter and targeting sequences, which are homologous to the 5' non-coding sequence of endogenous

polynucleotide sequence, flanking the promoter. The targeting sequence will be sufficiently near the 5' end of the polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination. The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter.

[1519] The amplified promoter and the amplified targeting sequences are digested with the appropriate restriction enzymes and subsequently treated with calf intestinal phosphatase. The digested promoter and digested targeting sequences are added together in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The construct is size fractionated on an agarose gel then purified by phenol extraction and ethanol precipitation.

[1520] In this Example, the polynucleotide constructs are administered as naked polynucleotides via electroporation. However, the polynucleotide constructs may also be administered with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, precipitating agents, etc. Such methods of delivery are known in the art.

[1521] Once the cells are transfected, homologous recombination will take place which results in the promoter being operably linked to the endogenous polynucleotide sequence. This results in the expression of polynucleotide corresponding to the polynucleotide in the cell. Expression may be detected by immunological staining, or any other method known in the art.

[1522] Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation. The supernatant is aspirated and the pellet is

resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2 HPO_4, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately $3X10^6$ cells/ml. Electroporation should be performed immediately following resuspension.

[1523] Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the locus corresponding to the polynucleotide of the invention, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI site on the 3'end. Two non-coding sequences are amplified via PCR: one non-coding sequence (fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3'end; the other non-coding sequence (fragment 2) is amplified with a BamHI site at the 5'end and a HindIII site at the 3'end. The CMV promoter and the fragments (1 and 2) are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; fragment 1 - XbaI; fragment 2 - BamHI) and ligated together. The resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid.

[1524] Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least 120 μg/ml. 0.5 ml of the cell suspension (containing approximately $1.5.X10^6$ cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at 960 μF and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

[1525] Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with 15% calf serum) in a 10 cm dish and incubated at 37 degree C. The following

day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

[1526] The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a patient as described above.

[1527] **Example 28: Method of Treatment Using Gene Therapy - In Vivo**

[1528] Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences into an animal to increase or decrease the expression of the polypeptide. The polynucleotide of the present invention may be operatively linked to a promoter or any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata et al., Cardiovasc. Res. 35(3):470-479 (1997); Chao et al., Pharmacol. Res. 35(6):517-522 (1997); Wolff, Neuromuscul. Disord. 7(5):314-318 (1997); Schwartz et al., Gene Ther. 3(5):405-411 (1996); Tsurumi et al., Circulation 94(12):3281-3290 (1996) (incorporated herein by reference).

[1529] The polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

[1530] The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the polynucleotides of the present invention may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al.

(1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

[1531] The polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

[1532] The polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

[1533] For the naked polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as

the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

[1534] The dose response effects of injected polynucleotide in muscle *in vivo* is determined as follows. Suitable template DNA for production of mRNA coding for polypeptide of the present invention is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

[1535] Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

[1536] After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for protein expression. A time course for protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other animals using naked

DNA.

[1537] Example 29: Transgenic Animals.

[1538] The polypeptides of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

[1539] Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pleuripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

[1540] Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

[1541] The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, *i.e.,* mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, *e.g.,* head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

[1542] Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

[1543] Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene on a distinct background that is appropriate for an experimental model of interest.

[1544] Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of polypeptides of the present invention, studying diseases, disorders, and/or conditions associated with aberrant expression, and in screening for compounds effective in ameliorating such diseases, disorders, and/or conditions.

[1545] Example 30: Knock-Out Animals.

[1546] Endogenous gene expression can also be reduced by inactivating or "knocking out" the gene and/or its promoter using targeted homologous recombination. (*E.g.*, see Smithies et al., Nature 317:230-234 (1985); Thomas & Capecchi, Cell 51:503-512 (1987); Thompson et al., Cell 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention *in vivo*. In another embodiment, techniques known in the art are used to generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination,

results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (*e.g.*, see Thomas & Capecchi 1987 and Thompson 1989, *supra*). However this approach can be routinely adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors that will be apparent to those of skill in the art.

[1547] In further embodiments of the invention, cells that are genetically engineered to express the polypeptides of the invention, or alternatively, that are genetically engineered not to express the polypeptides of the invention (e.g., knockouts) are administered to a patient *in vivo*. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered *in vitro* using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the polypeptides of the invention. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally.

[1548] Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. U.S. Patent No. 5,399,349; and

Mulligan & Wilson, U.S. Patent No. 5,460,959 each of which is incorporated by reference herein in its entirety).

[1549] When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

[1550] Transgenic and "knock-out" animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of polypeptides of the present invention, studying diseases, disorders, and/or conditions associated with aberrant expression, and in screening for compounds effective in ameliorating such diseases, disorders, and/or conditions.

[1551] **Example 31: Production of an Antibody**

[1552] Hybridoma Technology

[1553] The antibodies of the present invention can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, cells expressing polypeptide(s) of the invention are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of polypeptide(s) of the invention is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

[1554] Monoclonal antibodies specific for polypeptide(s) of the invention are prepared using hybridoma technology. (Kohler et al., Nature 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, an animal (preferably a mouse) is immunized with polypeptide(s) of the invention, or, more preferably, with a secreted polypeptide-expressing cell. Such polypeptide-expressing cells are cultured in any suitable tissue culture medium, preferably in Earle's modified Eagle's medium supplemented with

10% fetal bovine serum (inactivated at about 56°C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 μg/ml of streptomycin.

[1555] The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the polypeptide(s) of the invention.

[1556] Alternatively, additional antibodies capable of binding polypeptide(s) of the invention can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the polypeptide(s) of the invention protein-specific antibody can be blocked by polypeptide(s) of the invention. Such antibodies comprise anti-idiotypic antibodies to the polypeptide(s) of the invention protein-specific antibody and are used to immunize an animal to induce formation of further polypeptide(s) of the invention protein-specific antibodies.

[1557] For in vivo use of antibodies in humans, an antibody is "humanized". Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric and humanized antibodies are known in the art and are discussed herein. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., WO

8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985).)

[1558] Isolation Of Antibody Fragments Directed

[1559] polypeptide(s) of the invention From A Library Of scFvs

[1560] Naturally occurring V-genes isolated from human PBLs are constructed into a library of antibody fragments which contain reactivities against polypeptide(s) of the invention to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein by reference in its entirety).

[1561] Rescue of the Library. A library of scFvs is constructed from the RNA of human PBLs as described in PCT publication WO 92/01047. To rescue phage displaying antibody fragments, approximately 109 E. coli harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 μg/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to innoculate 50 ml of 2xTY-AMP-GLU, 2 x 108 TU of delta gene 3 helper (M13 delta gene III, see PCT publication WO 92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 μg/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in PCT publication WO 92/01047.

[1562] M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8,400 r.p.m. for 10 min), resuspended in 300 ml 2xTY broth containing 100 μg ampicillin/ml and 25 μg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al.,

1990), resuspended in 2 ml PBS and passed through a 0.45 μm filter (Minisart NML; Sartorius) to give a final concentration of approximately 1013 transducing units/ml (ampicillin-resistant clones).

[1563] Panning of the Library. Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 μg/ml or 10 μg/ml of a polypeptide of the present invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 1013 TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37°C. The E. coli are then plated on TYE plates containing 1% glucose and 100 μg/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

[1564] Characterization of Binders. Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., PCT publication WO 92/01047) and then by sequencing. These ELISA positive clones may also be further characterized by techniques known in the art, such as, for example, epitope mapping, binding affinity, receptor signal transduction, ability to block or competitively inhibit antibody/antigen binding, and competitive agonistic or antagonistic activity.

[1565] Example 32: Assays Detecting Stimulation or Inhibition of B cell Proliferation and Differentiation

[1566] Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B cell responsiveness including IL-2, IL-4, IL-5, IL-6, IL-7, IL10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce activation, proliferation, differentiation, homing, tolerance and death among B cell populations.

[1567] One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands CD154, CD70, and CD153 have been found to regulate a variety of immune responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of B-cell populations and their precursors.

[1568] In Vitro Assay- Purified polypeptides of the invention, or truncated forms thereof, is assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of the polypeptides of the invention on purified human tonsillar B cells, measured qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B cells are cultured in the presence of either formalin-fixed Staphylococcus aureus Cowan I (SAC) or immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B cells by magnetic bead (MACS) depletion of CD3-positive

cells. The resulting cell population is greater than 95% B cells as assessed by expression of CD45R(B220).

[1569] Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10^5 B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5 X 10^{-5}M 2ME, 100U/ml penicillin, 10ug/ml streptomycin, and 10^{-5} dilution of SAC) in a total volume of 150ul. Proliferation or inhibition is quantitated by a 20h pulse (1uCi/well) with 3H-thymidine (6.7 Ci/mM) beginning 72h post factor addition. The positive and negative controls are IL2 and medium respectively.

[1570] In Vivo Assay- BALB/c mice are injected (i.p.) twice per day with buffer only, or 2 mg/Kg of a polypeptide of the invention, or truncated forms thereof. Mice receive this treatment for 4 consecutive days, at which time they are sacrificed and various tissues and serum collected for analyses. Comparison of H&E sections from normal spleens and spleens treated with polypeptides of the invention identify the results of the activity of the polypeptides on spleen cells, such as the diffusion of peri-arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-cell populations. Immunohistochemical studies using a B cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell representation within loosely defined B-cell zones that infiltrate established T-cell regions.

[1571] Flow cytometric analyses of the spleens from mice treated with polypeptide is used to indicate whether the polypeptide specifically increases the proportion of ThB+, CD45R(B220)dull B cells over that which is observed in control mice.

[1572] Likewise, a predicted consequence of increased mature B-cell representation in vivo is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared between buffer and polypeptide-treated mice.

[1573] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

[1574] Example 33: T Cell Proliferation Assay

[1575] Proliferation assay for Resting PBLs.

[1576] A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of ^{3}H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 microliters per well of mAb to CD3 (HIT3a, Pharmingen) or isotype-matched control mAb (B33.1) overnight at 4°C (1 microgram/ml in .05M bicarbonate buffer, pH 9.5), then washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10^{4}/well) of mAb coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of TNF Delta and/or TNF Epsilon protein (total volume 200 microliters). Relevant protein buffer and medium alone are controls. After 48 hr. culture at 37°C, plates are spun for 2 min. at 1000 rpm and 100 microliters of supernatant is removed and stored -20°C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 microliters of medium containing 0.5 microcuries of ^{3}H-thymidine and cultured at 37°C for 18-24 hr. Wells are harvested and incorporation of ^{3}H-thymidine used as a measure of proliferation. Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances proliferation. Control antibody which does not induce proliferation of T cells is used as the negative controls for the effects of TNF Delta and/or TNF Epsilon proteins.

[1577] Alternatively, a proliferation assay on resting PBL (peripheral blood lymphocytes) is measured by the up-take of ^{3}H-thymidine. The assay is performed as follows. PBMC are isolated by Ficoll (LSM, ICN Biotechnologies, Aurora, Ohio) gradient centrifugation from human peripheral blood, and are cultured overnight in 10% (Fetal Calf Serum, Biofluids, Rockville, MD)/RPMI (Gibco BRL, Gaithersburg, MD). This overnight incubation period allows the adherent cells to attach to the plastic, which results in a lower background in the assay as there are fewer cells that can act as antigen presenting cells or that might be producing growth factors. The following day the non-adherent cells are collected, washed and used in the proliferation assay. The assay is performed in a 96 well plate using 2 x10^{4} cells/well in a final volume of 200 microliters. The supernatants (e.g., CHO or 293T supernatants) expressing the protein of interest are tested at a 30% final dilution, therefore 60ul are added to 140ul of 10% FCS/RPMI containing the cells. Control

supernatants are used at the same final dilution and express the following proteins: vector (negative control), IL-2 (*), IFNγ, TNFα, IL-10 and TR2. In addition to the control supernatants, recombinant human IL-2 (R & D Systems, Minneapolis, MN) at a final concentration of 100ng/ml is also used. After 24 hours of culture, each well is pulsed with 1uCi of ^{3}H-thymidine (Nen, Boston, MA). Cells are then harvested 20 hours following pulsing and incorporation of ^{3}H-thymidine is used as a measure of proliferation. Results are expressed as an average of triplicate samples plus or minus standard error.

(*) The amount of the control cytokines IL-2, IFNγ, TNFα and IL-10 produced in each transfection varies between 300pg to 5ng/ml.

[1578] Costimulation assay.

[1579] A costimulation assay on resting PBL (peripheral blood lymphocytes) is performed in the presence of immobilized antibodies to CD3 and CD28. The use of antibodies specific for the invariant regions of CD3 mimic the induction of T cell activation that would occur through stimulation of the T cell receptor by an antigen. Cross-linking of the TCR (first signal) in the absence of a costimulatory signal (second signal) causes very low induction of proliferation and will eventually result in a state of "anergy", which is characterized by the absence of growth and inability to produce cytokines. The addition of a costimulatory signal such as an antibody to CD28, which mimics the action of the costimulatory molecule. B7-1 expressed on activated APCs, results in enhancement of T cell responses including cell survival and production of IL-2. Therefore this type of assay allows to detect both positive and negative effects caused by addition of supernatants expressing the proteins of interest on T cell proliferation.

[1580] The assay is performed as follows. Ninety-six well plates are coated with 100ng/ml anti-CD3 and 5ug/ml anti-CD28 (Pharmingen, San Diego, CA) in a final volume of 100ul and incubated overnight at 4C. Plates are washed twice with PBS before use. PBMC are isolated by Ficoll (LSM, ICN Biotechnologies, Aurora, Ohio) gradient centrifugation from human peripheral blood, and are cultured overnight in 10% FCS(Fetal Calf Serum, Biofluids, Rockville, MD)/RPMI (Gibco BRL,

Gaithersburg, MD). This overnight incubation period allows the adherent cells to attach to the plastic, which results in a lower background in the assay as there are fewer cells that can act as antigen presenting cells or that might be producing growth factors. The following day the non adherent cells are collected, washed and used in the proliferation assay. The assay is performed in a 96 well plate using 2 $x10^4$ cells/well in a final volume of 200ul. The supernatants (e.g., CHO or 293T supernatants) expressing the protein of interest are tested at a 30% final dilution, therefore 60ul are added to 140ul of 10% FCS/RPMI containing the cells. Control supernatants are used at the same final dilution and express the following proteins: vector only (negative control), IL-2, IFNγ, TNFα, IL-10 and TR2. In addition to the control supernatants recombinant human IL-2 (R & D Systems, Minneapolis, MN) at a final concentration of 10ng/ml is also used. After 24 hours of culture, each well is pulsed with 1uCi of ^{3}H-thymidine (Nen, Boston, MA). Cells are then harvested 20 hours following pulsing and incorporation of ^{3}H-thymidine is used as a measure of proliferation. Results are expressed as an average of triplicate samples plus or minus standard error.

[1581] Proliferation assay for preactivated-resting T cells.

[1582] A proliferation assay on preactivated-resting T cells is performed on cells that are previously activated with the lectin phytohemagglutinin (PHA). Lectins are polymeric plant proteins that can bind to residues on T cell surface glycoproteins including the TCR and act as polyclonal activators. PBLs treated with PHA and then cultured in the presence of low doses of IL-2 resemble effector T cells. These cells are generally more sensitive to further activation induced by growth factors such as IL-2. This is due to the expression of high affinity IL-2 receptors that allows this population to respond to amounts of IL-2 that are 100 fold lower than what would have an effect on a naïve T cell. Therefore the use of this type of cells might enable to detect the effect of very low doses of an unknown growth factor, that would not be sufficient to induce proliferation on resting (naïve) T cells.

[1583] The assay is performed as follows. PBMC are isolated by F/H gradient centrifugation from human peripheral blood, and are cultured in10% FCS(Fetal Calf

Serum, Biofluids, Rockville, MD)/RPMI (Gibco BRL, Gaithersburg, MD) in the presence of 2ug/ml PHA (Sigma, Saint Louis, MO) for three days. The cells are then washed in PBS and cultured in10% FCS/RPMI in the presence of 5ng/ml of human recombinant IL-2 (R & D Systems, Minneapolis, MN) for 3 days. The cells are washed and rested in starvation medium (1%FCS/RPMI) for16 hours prior to the beginning of the proliferation assay. An aliquot of the cells is analyzed by FACS to determine the percentage of T cells (CD3 positive cells) present; this usually ranges between 93-97% depending on the donor. The assay is performed in a 96 well plate using 2 $x10^4$ cells/well in a final volume of 200ul. The supernatants (e.g., CHO or 293T supernatants) expressing the protein of interest are tested at a 30% final dilution, therefore 60ul are added to 140ul of in10% FCS/RPMI containing the cells. Control supernatants are used at the same final dilution and express the following proteins: vector (negative control), IL-2, IFNγ, TNFα, IL-10 and TR2. In addition to the control supernatants recombinant human IL-2 at a final concentration of 10ng/ml is also used. After 24 hours of culture, each well is pulsed with 1uCi of ^{3}H-thymidine(Nen, Boston, MA). Cells are then harvested 20 hours following pulsing and incorporation of ^{3}H-thymidine is used as a measure of proliferation. Results are expressed as an average of triplicate samples plus or minus standard error.

[1584] The studies described in this example test activity of polypeptides of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides of the invention (e.g., gene therapy), agonists, and/or antagonists of polynucleotides or polypeptides of the invention.

[1585] Example 34: Effect of Polypeptides of the Invention on the Expression of MHC Class II, Costimulatory and Adhesion Molecules and Cell Differentiation of Monocytes and Monocyte-Derived Human Dendritic Cells

[1586] Dendritic cells are generated by the expansion of proliferating precursors found in the peripheral blood: adherent PBMC or elutriated monocytic fractions are cultured for 7-10 days with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). These dendritic cells have the characteristic phenotype of immature cells (expression of CD1, CD80, CD86, CD40 and MHC class II antigens). Treatment with activating factors, such as TNF-α, causes a rapid

change in surface phenotype (increased expression of MHC class I and II, costimulatory and adhesion molecules, downregulation of FCγRII, upregulation of CD83). These changes correlate with increased antigen-presenting capacity and with functional maturation of the dendritic cells.

[1587] FACS analysis of surface antigens is performed as follows. Cells are treated 1-3 days with increasing concentrations of polypeptides of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

[1588] Effect on the production of cytokines. Cytokines generated by dendritic cells, in particular IL-12, are important in the initiation of T-cell dependent immune responses. IL-12 strongly influences the development of Thl helper T-cell immune response, and induces cytotoxic T and NK cell function. An ELISA is used to measure the IL-12 release as follows. Dendritic cells (10^6/ml) are treated with increasing concentrations of polypeptides of the invention for 24 hours. LPS (100 ng/ml) is added to the cell culture as positive control. Supernatants from the cell cultures are then collected and analyzed for IL-12 content using commercial ELISA kit (e..g, R & D Systems (Minneapolis, MN)). The standard protocols provided with the kits are used.

[1589] Effect on the expression of MHC Class II, costimulatory and adhesion molecules. Three major families of cell surface antigens can be identified on monocytes: adhesion molecules, molecules involved in antigen presentation, and Fc receptor. Modulation of the expression of MHC class II antigens and other costimulatory molecules, such as B7 and ICAM-1, may result in changes in the antigen presenting capacity of monocytes and ability to induce T cell activation. Increase expression of Fc receptors may correlate with improved monocyte cytotoxic activity, cytokine release and phagocytosis.

[1590] FACS analysis is used to examine the surface antigens as follows. Monocytes are treated 1-5 days with increasing concentrations of polypeptides of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal

antibodies for 30 minutes at 4 degreesC. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

[1591] Monocyte activation and/or increased survival. Assays for molecules that activate (or alternatively, inactivate) monocytes and/or increase monocyte survival (or alternatively, decrease monocyte survival) are known in the art and may routinely be applied to determine whether a molecule of the invention functions as an inhibitor or activator of monocytes. Polypeptides, agonists, or antagonists of the invention can be screened using the three assays described below. For each of these assays, Peripheral blood mononuclear cells (PBMC) are purified from single donor leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes are isolated from PBMC by counterflow centrifugal elutriation.

[1592] Monocyte Survival Assay. Human peripheral blood monocytes progressively lose viability when cultured in absence of serum or other stimuli. Their death results from internally regulated process (apoptosis). Addition to the culture of activating factors, such as TNF-alpha dramatically improves cell survival and prevents DNA fragmentation. Propidium iodide (PI) staining is used to measure apoptosis as follows. Monocytes are cultured for 48 hours in polypropylene tubes in serum-free medium (positive control), in the presence of 100 ng/ml TNF-alpha (negative control), and in the presence of varying concentrations of the compound to be tested. Cells are suspended at a concentration of 2 x 10^6/ml in PBS containing PI at a final concentration of 5 μg/ml, and then incubaed at room temperature for 5 minutes before FACScan analysis. PI uptake has been demonstrated to correlate with DNA fragmentation in this experimental paradigm.

[1593] Effect on cytokine release. An important function of monocytes/macrophages is their regulatory activity on other cellular populations of the immune system through the release of cytokines after stimulation. An ELISA to measure cytokine release is performed as follows. Human monocytes are incubated at a density of $5x10^5$ cells/ml with increasing concentrations of the a polypeptide of the invention and under the same conditions, but in the absence of the polypeptide. For IL-12 production, the cells are primed overnight with IFN (100 U/ml) in presence of a polypeptide of the invention. LPS (10 ng/ml) is then added. Conditioned media are collected after 24h and kept frozen until use. Measurement of TNF-alpha, IL-10, MCP-1 and IL-8 is then performed using a

commercially available ELISA kit (e..g, R & D Systems (Minneapolis, MN)) and applying the standard protocols provided with the kit.

[1594] Oxidative burst. Purified monocytes are plated in 96-w plate at 2-1x10^5 cell/well. Increasing concentrations of polypeptides of the invention are added to the wells in a total volume of 0.2 ml culture medium (RPMI 1640 + 10% FCS, glutamine and antibiotics). After 3 days incubation, the plates are centrifuged and the medium is removed from the wells. To the macrophage monolayers, 0.2 ml per well of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of HRPO) is added, together with the stimulant (200 nM PMA). The plates are incubated at 37°C for 2 hours and the reaction is stopped by adding 20 μl 1N NaOH per well. The absorbance is read at 610 nm. To calculate the amount of H_2O_2 produced by the macrophages, a standard curve of a H_2O_2 solution of known molarity is performed for each experiment.

[1595] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polypeptides, polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1596] Example 35: Biological Effects of Polypeptides of the Invention

[1597] Astrocyte and Neuronal Assays·

[1598] Recombinant polypeptides of the invention, expressed in *Escherichia coli* and purified as described above, can be tested for activity in promoting the survival, neurite outgrowth, or phenotypic differentiation of cortical neuronal cells and for inducing the proliferation of glial fibrillary acidic protein immunopositive cells, astrocytes. The selection of cortical cells for the bioassay is based on the prevalent expression of FGF-1 and FGF-2 in cortical structures and on the previously reported enhancement of cortical neuronal survival resulting from FGF-2 treatment. A thymidine incorporation assay, for example, can be used to elucidate a polypeptide of the invention's activity on these cells.

[1599] Moreover, previous reports describing the biological effects of FGF-2 (basic FGF) on cortical or hippocampal neurons *in vitro* have demonstrated increases in both neuron

survival and neurite outgrowth (Walicke et al., "Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension." *Proc. Natl. Acad. Sci. USA 83*:3012-3016. (1986), assay herein incorporated by reference in its entirety). However, reports from experiments done on PC-12 cells suggest that these two responses are not necessarily synonymous and may depend on not only which FGF is being tested but also on which receptor(s) are expressed on the target cells. Using the primary cortical neuronal culture paradigm, the ability of a polypeptide of the invention to induce neurite outgrowth can be compared to the response achieved with FGF-2 using, for example, a thymidine incorporation assay.

[1600] Fibroblast and endothelial cell assays·

[1601] Human lung fibroblasts are obtained from Clonetics (San Diego, CA) and maintained in growth media from Clonetics. Dermal microvascular endothelial cells are obtained from Cell Applications (San Diego, CA). For proliferation assays, the human lung fibroblasts and dermal microvascular endothelial cells can be cultured at 5,000 cells/well in a 96-well plate for one day in growth medium. The cells are then incubated for one day in 0.1% BSA basal medium. After replacing the medium with fresh 0.1% BSA medium, the cells are incubated with the test proteins for 3 days. Alamar Blue (Alamar Biosciences, Sacramento, CA) is added to each well to a final concentration of 10%. The cells are incubated for 4 hr. Cell viability is measured by reading in a CytoFluor fluorescence reader. For the PGE_2 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or polypeptides of the invention with or without IL-1α for 24 hours. The supernatants are collected and assayed for PGE_2 by EIA kit (Cayman, Ann Arbor, MI). For the IL-6 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or with or without polypeptides of the invention IL-1α for 24 hours. The supernatants are collected and assayed for IL-6 by ELISA kit (Endogen, Cambridge, MA).

[1602] Human lung fibroblasts are cultured with FGF-2 or polypeptides of the invention for 3 days in basal medium before the addition of Alamar Blue to assess effects on growth of the fibroblasts. FGF-2 should show a stimulation at 10 - 2500 ng/ml which can be used to compare stimulation with polypeptides of the invention.

[1603] Parkinson Models.

[1604] The loss of motor function in Parkinson's disease is attributed to a deficiency of striatal dopamine resulting from the degeneration of the nigrostriatal dopaminergic projection neurons. An animal model for Parkinson's that has been extensively characterized involves the systemic administration of 1-methyl-4 phenyl 1,2,3,6-tetrahydropyridine (MPTP). In the CNS, MPTP is taken-up by astrocytes and catabolized by monoamine oxidase B to 1-methyl-4-phenyl pyridine (MPP^+) and released. Subsequently, MPP^+ is actively accumulated in dopaminergic neurons by the high-affinity reuptake transporter for dopamine. MPP^+ is then concentrated in mitochondria by the electrochemical gradient and selectively inhibits nicotidamide adenine disphosphate: ubiquinone oxidoreductionase (complex I), thereby interfering with electron transport and eventually generating oxygen radicals.

[1605] It has been demonstrated in tissue culture paradigms that FGF-2 (basic FGF) has trophic activity towards nigral dopaminergic neurons (Ferrari et al., Dev. Biol. 1989). Recently, Dr. Unsicker's group has demonstrated that administering FGF-2 in gel foam implants in the striatum results in the near complete protection of nigral dopaminergic neurons from the toxicity associated with MPTP exposure (Otto and Unsicker, J. Neuroscience, 1990).

[1606] Based on the data with FGF-2, polypeptides of the invention can be evaluated to determine whether it has an action similar to that of FGF-2 in enhancing dopaminergic neuronal survival *in vitro* and it can also be tested *in vivo* for protection of dopaminergic neurons in the striatum from the damage associated with MPTP treatment. The potential effect of a polypeptide of the invention is first examined in vitro in a dopaminergic neuronal cell culture paradigm. The cultures are prepared by dissecting the midbrain floor plate from gestation day 14 Wistar rat embryos. The tissue is dissociated with trypsin and seeded at a density of 200,000 cells/cm^2 on polyorthinine-laminin coated glass coverslips. The cells are maintained in Dulbecco's Modified Eagle's medium and F12 medium

containing hormonal supplements (N1). The cultures are fixed with paraformaldehyde after 8 days in vitro and are processed for tyrosine hydroxylase, a specific marker for dopminergic neurons, immunohistochemical staining. Dissociated cell cultures are prepared from embryonic rats. The culture medium is changed every third day and the factors are also added at that time.

[1607] Since the dopaminergic neurons are isolated from animals at gestation day 14, a developmental time which is past the stage when the dopaminergic precursor cells are proliferating, an increase in the number of tyrosine hydroxylase immunopositive neurons would represent an increase in the number of dopaminergic neurons surviving *in vitro*. Therefore, if a polypeptide of the invention acts to prolong the survival of dopaminergic neurons, it would suggest that the polypeptide may be involved in Parkinson's Disease.

[1608] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1609] Example 36: The Effect of Polypeptides of the Invention on the Growth of Vascular Endothelial Cells

[1610] On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at 2-5x10^4 cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnique, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin. A polypeptide having the amino acid sequence of SEQ ID NO:Y, and positive controls, such as VEGF and basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

[1611] An increase in the number of HUVEC cells indicates that the polypeptide of the invention may proliferate vascular endothelial cells.

[1612] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to

test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1613] Example 37: Stimulatory Effect of Polypeptides of the Invention on the Proliferation of Vascular Endothelial Cells

[1614] For evaluation of mitogenic activity of growth factors, the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay with the electron coupling reagent PMS (phenazine methosulfate) was performed (CellTiter 96 AQ, Promega). Cells are seeded in a 96-well plate (5,000 cells/well) in 0.1 mL serum-supplemented medium and are allowed to attach overnight. After serum-starvation for 12 hours in 0.5% FBS, conditions (bFGF, $VEGF_{165}$ or a polypeptide of the invention in 0.5% FBS) with or without Heparin (8 U/ml) are added to wells for 48 hours. 20 mg of MTS/PMS mixture (1:0.05) are added per well and allowed to incubate for 1 hour at 37°C before measuring the absorbance at 490 nm in an ELISA plate reader. Background absorbance from control wells (some media, no cells) is subtracted, and seven wells are performed in parallel for each condition. See, Leak *et al. In Vitro Cell. Dev. Biol. 30A:*512-518 (1994).

[1615] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1616] Example 38: Inhibition of PDGF-induced Vascular Smooth Muscle Cell Proliferation Stimulatory Effect

[1617] HAoSMC proliferation can be measured, for example, by BrdUrd incorporation. Briefly, subconfluent, quiescent cells grown on the 4-chamber slides are transfected with CRP or FITC-labeled AT2-3LP. Then, the cells are pulsed with 10% calf serum and 6 mg/ml BrdUrd. After 24 h, immunocytochemistry is performed by using BrdUrd Staining Kit (Zymed Laboratories). In brief, the cells are incubated with the biotinylated mouse anti-BrdUrd antibody at 4 degrees C for 2 h after being exposed to denaturing solution and then incubated with the streptavidin-peroxidase and diaminobenzidine. After counterstaining with hematoxylin, the cells are mounted for microscopic examination, and

the BrdUrd-positive cells are counted. The BrdUrd index is calculated as a percent of the BrdUrd-positive cells to the total cell number. In addition, the simultaneous detection of the BrdUrd staining (nucleus) and the FITC uptake (cytoplasm) is performed for individual cells by the concomitant use of bright field illumination and dark field-UV fluorescent illumination. See, Hayashida et al., J. Biol. Chem. 6:271(36):21985-21992 (1996).

[1618] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1619] **Example 39: Stimulation of Endothelial Migration**

[1620] This example will be used to explore the possibility that a polypeptide of the invention may stimulate lymphatic endothelial cell migration.

[1621] Endothelial cell migration assays are performed using a 48 well microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD; Falk, W., et al., J. Immunological Methods 1980;33:239-247). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8 um (Nucleopore Corp. Cambridge, MA) are coated with 0.1% gelatin for at least 6 hours at room temperature and dried under sterile air. Test substances are diluted to appropriate concentrations in M199 supplemented with 0.25% bovine serum albumin (BSA), and 25 ul of the final dilution is placed in the lower chamber of the modified Boyden apparatus. Subconfluent, early passage (2-6) HUVEC or BMEC cultures are washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between lower and upper chamber, 2.5 x 10^5 cells suspended in 50 ul M199 containing 1% FBS are seeded in the upper compartment. The apparatus is then incubated for 5 hours at 37°C in a humidified chamber with 5% CO2 to allow cell migration. After the incubation period, the filter is removed and the upper side of the filter with the non-migrated cells is scraped with a rubber policeman. The filters are fixed with methanol and stained with a Giemsa solution (Diff-Quick, Baxter, McGraw Park, IL). Migration is quantified by counting cells of three random high-power fields (40x) in each well, and all groups are performed in quadruplicate.

[1622] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1623] **Example 40: Stimulation of Nitric Oxide Production by Endothelial Cells**

[1624] Nitric oxide released by the vascular endothelium is believed to be a mediator of vascular endothelium relaxation. Thus, activity of a polypeptide of the invention can be assayed by determining nitric oxide production by endothelial cells in response to the polypeptide.

[1625] Nitric oxide is measured in 96-well plates of confluent microvascular endothelial cells after 24 hours starvation and a subsequent 4 hr exposure to various levels of a positive control (such as VEGF-1) and the polypeptide of the invention. Nitric oxide in the medium is determined by use of the Griess reagent to measure total nitrite after reduction of nitric oxide-derived nitrate by nitrate reductase. The effect of the polypeptide of the invention on nitric oxide release is examined on HUVEC.

[1626] Briefly, NO release from cultured HUVEC monolayer is measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments Inc.) (1049). Calibration of the NO elements is performed according to the following equation:

$$2\ KNO_2 + 2\ KI + 2\ H_2SO_4\ 6\ 2\ NO + I_2 + 2\ H_2O + 2\ K_2SO_4$$

[1627] The standard calibration curve is obtained by adding graded concentrations of KNO_2 (0, 5, 10, 25, 50, 100, 250, and 500 nmol/L) into the calibration solution containing KI and H_2SO_4. The specificity of the Iso-NO electrode to NO is previously determined by measurement of NO from authentic NO gas (1050). The culture medium is removed and HUVECs are washed twice with Dulbecco's phosphate buffered saline. The cells are then bathed in 5 ml of filtered Krebs-Henseleit solution in 6-well plates, and the cell plates are kept on a slide warmer (Lab Line Instruments Inc.) To maintain the temperature at 37°C. The NO sensor probe is inserted vertically into the wells, keeping the tip of the electrode 2 mm under the surface of the solution, before addition of the different conditions. S-nitroso acetyl penicillamin (SNAP) is used as a positive control. The amount of

released NO is expressed as picomoles per $1x10^6$ endothelial cells. All values reported are means of four to six measurements in each group (number of cell culture wells). See, Leak *et al. Biochem. and Biophys. Res. Comm. 217*:96-105 (1995).

[1628] The studies described in this example tested activity of polypeptides of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1629] Example 41: Effect of Polypepides of the Invention on Cord Formation in Angiogenesis

[1630] Another step in angiogenesis is cord formation, marked by differentiation of endothelial cells. This bioassay measures the ability of microvascular endothelial cells to form capillary-like structures (hollow structures) when cultured *in vitro*.

[1631] CADMEC (microvascular endothelial cells) are purchased from Cell Applications, Inc. as proliferating (passage 2) cells and are cultured in Cell Applications' CADMEC Growth Medium and used at passage 5. For the *in vitro* angiogenesis assay, the wells of a 48-well cell culture plate are coated with Cell Applications' Attachment Factor Medium (200 ml/well) for 30 min. at 37°C. CADMEC are seeded onto the coated wells at 7,500 cells/well and cultured overnight in Growth Medium. The Growth Medium is then replaced with 300 mg Cell Applications' Chord Formation Medium containing control buffer or a polypeptide of the invention (0.1 to 100 ng/ml) and the cells are cultured for an additional 48 hr. The numbers and lengths of the capillary-like chords are quantitated through use of the Boeckeler VIA-170 video image analyzer. All assays are done in triplicate.

[1632] Commercial (R&D) VEGF (50 ng/ml) is used as a positive control. b-esteradiol (1 ng/ml) is used as a negative control. The appropriate buffer (without protein) is also utilized as a control.

[1633] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1634] Example 42: Angiogenic Effect on Chick Chorioallantoic Membrane

[1635] Chick chorioallantoic membrane (CAM) is a well-established system to examine angiogenesis. Blood vessel formation on CAM is easily visible and quantifiable. The ability of polypeptides of the invention to stimulate angiogenesis in CAM can be examined.

[1636] Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese qual (*Coturnix coturnix*) are incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old qual embryos is studied with the following methods.

[1637] On Day 4 of development, a window is made into the egg shell of chick eggs. The embryos are checked for normal development and the eggs sealed with cellotape. They are further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) are cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors are dissolved in distilled water and about 3.3 mg/ 5 ml are pipetted on the disks. After air-drying, the inverted disks are applied on CAM. After 3 days, the specimens are fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They are photographed with a stereo microscope [Wild M8] and embedded for semi- and ultrathin sectioning as described above. Controls are performed with carrier disks alone.

[1638] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1639] Example 43: Angiogenesis Assay Using a Matrigel Implant in Mouse

[1640] *In vivo* angiogenesis assay of a polypeptide of the invention measures the ability of an existing capillary network to form new vessels in an implanted capsule of murine extracellular matrix material (Matrigel). The protein is mixed with the liquid Matrigel at 4 degree C and the mixture is then injected subcutaneously in mice where it solidifies. After 7 days, the solid "plug" of Matrigel is removed and examined for the presence of new blood vessels. Matrigel is purchased from Becton Dickinson Labware/Collaborative Biomedical Products.

[1641] When thawed at 4 degree C the Matrigel material is a liquid. The Matrigel is mixed with a polypeptide of the invention at 150 ng/ml at 4 degrees C and drawn into cold 3 ml syringes. Female C57Bl/6 mice approximately 8 weeks old are injected with the mixture of Matrigel and experimental protein at 2 sites at the midventral aspect of the abdomen (0.5 ml/site). After 7 days, the mice are sacrificed by cervical dislocation, the Matrigel plugs are removed and cleaned (i.e., all clinging membranes and fibrous tissue is removed). Replicate whole plugs are fixed in neutral buffered 10% formaldehyde, embedded in paraffin and used to produce sections for histological examination after staining with Masson's Trichrome. Cross sections from 3 different regions of each plug are processed. Selected sections are stained for the presence of vWF. The positive control for this assay is bovine basic FGF (150 ng/ml). Matrigel alone is used to determine basal levels of angiogenesis.

[1642] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1643] Example 44: Rescue of Ischemia in Rabbit Lower Limb Model

[1644] To study the in vivo effects of polynucleotides and polypeptides of the invention on ischemia, a rabbit hindlimb ischemia model is created by surgical removal of one femoral arteries as described previously (Takeshita *et al.*, *Am J. Pathol 147*:1649-1660 (1995)). The excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels originating from the internal iliac artery (Takeshita*et al. Am J. Pathol 147*:1649-1660 (1995)). An interval of 10 days is allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. At 10 day post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb is transfected with 500 mg naked expression plasmid containing a polynucleotide of the invention by arterial gene transfer technology using a hydrogel-coated balloon catheter as described (Riessen *et al. Hum Gene Ther. 4*:749-758

(1993); Leclerc *et al. J. Clin. Invest. 90*: 936-944 (1992)). When a polypeptide of the invention is used in the treatment, a single bolus of 500 mg polypeptide of the invention or control is delivered into the internal iliac artery of the ischemic limb over a period of 1 min. through an infusion catheter. On day 30, various parameters are measured in these rabbits: (a) BP ratio - The blood pressure ratio of systolic pressure of the ischemic limb to that of normal limb; (b) Blood Flow and Flow Reserve - Resting FL: the blood flow during undilated condition and Max FL: the blood flow during fully dilated condition (also an indirect measure of the blood vessel amount) and Flow Reserve is reflected by the ratio of max FL: resting FL; (c) Angiographic Score - This is measured by the angiogram of collateral vessels. A score is determined by the percentage of circles in an overlaying grid that with crossing opacified arteries divided by the total number m the rabbit thigh; (d) Capillary density - The number of collateral capillaries determined in light microscopic sections taken from hindlimbs.

[1645] The studies described in this example tested activity of polynucleotides and polypeptides of the invention. However, one skilled in the art could easily modify the exemplified studies to test the agonists, and/or antagonists of the invention.

[1646] **Example 45: Effect of Polypeptides of the Invention on Vasodilation**

[1647] Since dilation of vascular endothelium is important in reducing blood pressure, the ability of polypeptides of the invention to affect the blood pressure in spontaneously hypertensive rats (SHR) is examined. Increasing doses (0, 10, 30, 100, 300, and 900 mg/kg) of the polypeptides of the invention are administered to 13-14 week old spontaneously hypertensive rats (SHR). Data are expressed as the mean +/- SEM. Statistical analysis are performed with a paired t-test and statistical significance is defined as $p<0.05$ vs. the response to buffer alone.

[1648] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1649] Example 46: Rat Ischemic Skin Flap Model

[1650] The evaluation parameters include skin blood flow, skin temperature, and factor VIII immunohistochemistry or endothelial alkaline phosphatase reaction. Expression of polypeptides of the invention, during the skin ischemia, is studied using in situ hybridization.

[1651] The study in this model is divided into three parts as follows:

a) Ischemic skin

b) Ischemic skin wounds

c) Normal wounds

[1652] The experimental protocol includes:

a) Raising a 3x4 cm, single pedicle full-thickness random skin flap (myocutaneous flap over the lower back of the animal).

b) An excisional wounding (4-6 mm in diameter) in the ischemic skin (skin-flap).

c) Topical treatment with a polypeptide of the invention of the excisional wounds (day 0, 1, 2, 3, 4 post-wounding) at the following various dosage ranges: 1mg to 100 mg.

d) Harvesting the wound tissues at day 3, 5, 7, 10, 14 and 21 post-wounding for histological, immunohistochemical, and in situ studies.

[1653] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1654] Example 47: Peripheral Arterial Disease Model

[1655] Angiogenic therapy using a polypeptide of the invention is a novel therapeutic strategy to obtain restoration of blood flow around the ischemia in case of peripheral arterial diseases. The experimental protocol includes:

a) One side of the femoral artery is ligated to create ischemic muscle of the hindlimb, the other side of hindlimb serves as a control.

b) a polypeptide of the invention, in a dosage range of 20 mg - 500 mg, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-3 weeks.

c) The ischemic muscle tissue is collected after ligation of the femoral artery at 1, 2, and 3 weeks for the analysis of expression of a polypeptide of the invention and histology. Biopsy is also performed on the other side of normal muscle of the contralateral hindlimb.

[1656] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1657] Example 48: Ischemic Myocardial Disease Model

[1658] A polypeptide of the invention is evaluated as a potent mitogen capable of stimulating the development of collateral vessels, and restructuring new vessels after coronary artery occlusion. Alteration of expression of the polypeptide is investigated in situ. The experimental protocol includes:

a) The heart is exposed through a left-side thoracotomy in the rat. Immediately, the left coronary artery is occluded with a thin suture (6-0) and the thorax is closed.

b) a polypeptide of the invention, in a dosage range of 20 mg - 500 mg, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-4 weeks.

c) Thirty days after the surgery, the heart is removed and cross-sectioned for morphometric and in situ analyzes.

[1659] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1660] Example 49: Rat Corneal Wound Healing Model

[1661] This animal model shows the effect of a polypeptide of the invention on neovascularization. The experimental protocol includes:

a) Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.

b) Inserting a spatula below the lip of the incision facing the outer corner of the eye.

c) Making a pocket (its base is 1-1.5 mm form the edge of the eye).

d) Positioning a pellet, containing 50ng- 5ug of a polypeptide of the invention, within the pocket.

e) Treatment with a polypeptide of the invention can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

[1662] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1663] Example 50: Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models

[1664] ***A. Diabetic db+/db+ Mouse Model.***

[1665] To demonstrate that a polypeptide of the invention accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al., J. Surg. Res. 52*:389 (1992); Greenhalgh, D.G. *et al., Am. J. Pathol. 136*:1235 (1990)).

[1666] The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al. Proc. Natl. Acad. Sci. USA 77*:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al., J. Immunol. 120*:1375 (1978); Debray-Sachs, M. *et al., Clin. Exp. Immunol. 51(1)*:1-7 (1983); Leiter *et al., Am.*

J. of Pathol. 114:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al., Exp. Neurol. 83(2)*:221-232 (1984); Robertson *et al., Diabetes 29(1)*:60-67 (1980); Giacomelli *et al., Lab Invest. 40(4)*:460-473 (1979); Coleman, D.L., *Diabetes 31 (Suppl)*:1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al., J. Immunol. 120*:1375-1377 (1978)).

[1667] The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al., Am. J. of Pathol. 136*:1235-1246 (1990)).

[1668] Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

[1669] Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med. 172*:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

[1670] Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily

measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

[1671] A polypeptide of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

[1672] Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

[1673] Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) untreated group, and 3) treated group.

[1674] Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is $64mm^2$, the corresponding size of the dermal punch. Calculations are made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

[1675] Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with a polypeptide of the invention. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al., Am. J. Pathol. 136:*1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

[1676] Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a

positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

[1677] Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer can serve as a positive tissue control and human brain tissue can be used as a negative tissue control. Each specimen includes a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

[1678] Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

[1679] ***B. Steroid Impaired Rat Model***

[1680] The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl*et al., J. Immunol. 115*: 476-481 (1975); Werb *et al., J. Exp. Med. 147*:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert *et al., An. Intern. Med. 37*:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck *et al., Growth Factors. 5:* 295-304 (1991); Haynes *et al., J. Clin. Invest. 61:* 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes et al., *J. Clin. Invest. 61:* 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In:* Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck *et al., Growth Factors. 5:* 295-304 (1991); Haynes *et al., J. Clin. Invest. 61:* 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In*: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce *et al., Proc. Natl. Acad. Sci. USA 86*: 2229-2233 (1989)).

[1681] To demonstrate that a polypeptide of the invention can accelerate the healing process, the effects of multiple topical applications of the polypeptide on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

[1682] Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

[1683] The wounding protocol is followed according to section A, above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

[1684] Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

[1685] The polypeptide of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

[1686] Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

[1687] Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated: 1) Untreated group 2) Vehicle placebo control 3) treated groups.

[1688] Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is $64mm^2$, the corresponding size of the dermal punch. Calculations are made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

[1689] Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with a polypeptide of the invention. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

[1690] Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

[1691] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1692] **Example 51: Lymphadema Animal Model**

[1693] The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of a polypeptide of the

invention in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

[1694] Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

[1695] Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated suture ligated.

[1696] Using a microscope, muscles in back of the leg (near the semitendinosis and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then and ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

[1697] Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

[1698] To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which

typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

[1699] Circumference Measurements: Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people then those 2 readings are averaged. Readings are taken from both control and edematous limbs.

[1700] Volumetric Measurements: On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

[1701] Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and Ca2+ comparison.

[1702] Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs are amputated using a quillitine, then both experimental and control legs are cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

[1703] Histological Preparations: The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle is observed under fluorescent microscopy for lymphatics..

[1704] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1705] Example 52: Suppression of TNF alpha-induced adhesion molecule expression by a Polypeptide of the Invention

[1706] The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

[1707] Tumor necrosis factor alpha (TNF-a), a potent proinflammatory cytokine, is a stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

[1708] The potential of a polypeptide of the invention to mediate a suppression of TNF-a induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM expression on TNF-a treated ECs when co-stimulated with a member of the FGF family of proteins.

[1709] To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2; Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO_2. HUVECs are seeded in 96-well plates at concentrations of 1 x 10^4 cells/well in EGM medium at 37 degree C for 18-24 hrs or until confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given cytokine and/or growth factor(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

[1710] Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well plate to confluence. Growth medium is removed from the cells and replaced with 90 ul of 199 Medium (10% FBS). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10 ul volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μl of 0.1% paraformaldehyde-PBS(with Ca++ and Mg++) is added to each well. Plates are held at 4°C for 30 min.

[1711] Fixative is then removed from the wells and wells are washed 1X with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10 μl of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μg/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA.

[1712] Then add 20 μl of diluted ExtrAvidin-Alkaline Phosphotase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH 10.4). 100 μl of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphotase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μl of pNNP reagent must then be added to each of the standard wells. The plate must be incubated at 37°C for 4h. A volume of 50 μl of 3M NaOH is added to all wells. The results are quantified on a plate reader at 405 nm. The background subtraction option is used on blank wells filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

[1713] The studies described in this example tested activity of a polypeptide of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), agonists, and/or antagonists of the invention.

[1714] Example 53: Assay for the Stimulation of Bone Marrow CD34+ Cell Proliferation

[1715] This assay is based on the ability of human CD34+ to proliferate in the presence of hematopoietic growth factors and evaluates the ability of isolated polypeptides expressed in mammalian cells to stimulate proliferation of CD34+ cells.
[1716] It has been previously shown that most mature precursors will respond to only a single signal. More immature precursors require at least two signals to respond. Therefore, to test the effect of polypeptides on hematopoietic activity of a wide range of progenitor cells, the assay contains a given polypeptide in the presence or absence of other hematopoietic growth factors. Isolated cells are cultured for 5 days in the presence of Stem Cell Factor (SCF) in combination with tested sample. SCF alone has a very limited effect on the proliferation of bone marrow (BM) cells, acting in such conditions only as a "survival" factor. However, combined with any factor exhibiting stimulatory effect on these cells (e.g., IL-3), SCF will cause a synergistic effect. Therefore, if the tested polypeptide has a stimulatory effect on a hematopoietic progenitors, such activity can be easily detected. Since normal BM cells have a low level of cycling cells, it is likely that any inhibitory effect of a given polypeptide, or agonists or antagonists thereof, might not be detected. Accordingly, assays for an inhibitory effect on progenitors is preferably tested in cells that are first subjected to *in vitro* stimulation with SCF+IL+3, and then contacted with the compound that is being evaluated for inhibition of such induced proliferation.
[1717] Briefly, CD34+ cells are isolated using methods known in the art. The cells are thawed and resuspended in medium (QBSF 60 serum-free medium with 1% L-glutamine (500ml) Quality Biological, Inc., Gaithersburg, MD Cat# 160-204-101). After several gentle centrifugation steps at 200 x g, cells are allowed to rest for one hour. The cell count is adjusted to 2.5 x 10^5 cells/ml. During this time, 100 µl of sterile water is added to the peripheral wells of a 96-well plate. The cytokines that can be tested with a given polypeptide in this assay is rhSCF (R&D Systems, Minneapolis, MN, Cat# 255-SC) at 50 ng/ml alone and in combination with rhSCF

and rhIL-3 (R&D Systems, Minneapolis, MN, Cat# 203-ML) at 30 ng/ml. After one hour, 10 μl of prepared cytokines, 50 μl SID (supernatants at 1:2 dilution = 50 μl) and 20 μl of diluted cells are added to the media which is already present in the wells to allow for a final total volume of 100 μl. The plates are then placed in a 37°C/5% CO_2 incubator for five days.

[1718] Eighteen hours before the assay is harvested, 0.5 μCi/well of [3H] Thymidine is added in a 10 μl volume to each well to determine the proliferation rate. The experiment is terminated by harvesting the cells from each 96-well plate to a filtermat using the Tomtec Harvester 96. After harvesting, the filtermats are dried, trimmed and placed into OmniFilter assemblies consisting of one OmniFilter plate and one OmniFilter Tray. 60 μl Microscint is added to each well and the plate sealed with TopSeal-A press-on sealing film A bar code 15 sticker is affixed to the first plate for counting. The sealed plates is then loaded and the level of radioactivity determined via the Packard Top Count and the printed data collected for analysis. The level of radioactivity reflects the amount of cell proliferation.

[1719] The studies described in this example test the activity of a given polypeptide to stimulate bone marrow CD34+ cell proliferation. One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), antibodies, agonists, and/or antagonists and fragments and variants thereof. As a nonlimiting example, potential antagonists tested in this assay would be expected to inhibit cell proliferation in the presence of cytokines and/or to increase the inhibition of cell proliferation in the presence of cytokines and a given polypeptide. In contrast, potential agonists tested in this assay would be expected to enhance cell proliferation and/or to decrease the inhibition of cell proliferation in the presence of cytokines and a given polypeptide.

[1720] The ability of a gene to stimulate the proliferation of bone marrow CD34+ cells indicates that polynucleotides and polypeptides corresponding to the gene are useful for the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein.

[1721] Example 54: Assay for Extracellular Matrix Enhanced Cell Response (EMECR)

[1722] The objective of the Extracellular Matrix Enhanced Cell Response (EMECR) assay is to identify gene products (e.g., isolated polypeptides) that act on the hematopoietic stem cells in the context of the extracellular matrix (ECM) induced signal.

[1723] Cells respond to the regulatory factors in the context of signal(s) received from the surrounding microenvironment. For example, fibroblasts, and endothelial and epithelial stem cells fail to replicate in the absence of signals from the ECM. Hematopoietic stem cells can undergo self-renewal in the bone marrow, but not in *in vitro* suspension culture. The ability of stem cells to undergo self-renewal *in vitro* is dependent upon their interaction with the stromal cells and the ECM protein fibronectin (fn). Adhesion of cells to fn is mediated by the $\alpha_5.\beta_1$ and $\alpha_4.\beta_1$ integrin receptors, which are expressed by human and mouse hematopoietic stem cells. The factor(s) which integrate with the ECM environment and responsible for stimulating stem cell self-renewal has not yet been identified. Discovery of such factors should be of great interest in gene therapy and bone marrow transplant applications

[1724] Briefly, polystyrene, non tissue culture treated, 96-well plates are coated with fn fragment at a coating concentration of 0.2 $\mu g/ cm^2$. Mouse bone marrow cells are plated (1,000 cells/well) in 0.2 ml of serum-free medium. Cells cultured in the presence of IL-3 (5 ng/ml) + SCF (50 ng/ml) would serve as the positive control, conditions under which little self-renewal but pronounced differentiation of the stem cells is to be expected. Gene products are tested with appropriate negative controls in the presence and absence of SCF(5.0 ng/ml), where test factor supernates represent 10% of the total assay volume. The plated cells are then allowed to grow by incubating in a low oxygen environment (5% CO_2, 7% O_2, and 88% N_2) tissue culture incubator for 7 days. The number of proliferating cells within the wells is then quantitated by measuring thymidine incorporation into cellular DNA. Verification of the positive hits in the assay will require phenotypic characterization of the cells, which can be accomplished by scaling up of the culture system and using appropriate antibody reagents against cell surface antigens and FACScan.

[1725] One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), antibodies, agonists, and/or antagonists and fragments and variants thereof.

[1726] If a particular gene product is found to be a stimulator of hematopoietic progenitors, polynucleotides and polypeptides corresponding to the gene may be useful for the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein. The gene product may also be useful in the expansion of stem cells and committed progenitors of various blood lineages, and in the differentiation and/or proliferation of various cell types.

[1727] Additionally, the polynucleotides and/or polypeptides of the gene of interest and/or agonists and/or antagonists thereof, may also be employed to inhibit the proliferation and differentiation of hematopoietic cells and therefore may be employed to protect bone marrow stem cells from chemotherapeutic agents during chemotherapy. This antiproliferative effect may allow administration of higher doses of chemotherapeutic agents and, therefore, more effective chemotherapeutic treatment.

[1728] Moreover, polynucleotides and polypeptides corresponding to the gene of interest may also be useful for the treatment and diagnosis of hematopoietic related disorders such as, for example, anemia, pancytopenia, leukopenia, thrombocytopenia or leukemia since stromal cells are important in the production of cells of hematopoietic lineages. The uses include bone marrow cell ex-vivo culture, bone marrow transplantation, bone marrow reconstitution, radiotherapy or chemotherapy of neoplasia.

[1729] Example 55: Human Dermal Fibroblast and Aortic Smooth Muscle Cell Proliferation

[1730] The polypeptide of interest is added to cultures of normal human dermal fibroblasts (NHDF) and human aortic smooth muscle cells (AoSMC) and two co-assays are performed with each sample. The first assay examines the effect of the polypeptide of interest on the proliferation of normal human dermal fibroblasts

(NHDF) or aortic smooth muscle cells (AoSMC). Aberrant growth of fibroblasts or smooth muscle cells is a part of several pathological processes, including fibrosis, and restenosis. The second assay examines IL6 production by both NHDF and SMC. IL6 production is an indication of functional activation. Activated cells will have increased production of a number of cytokines and other factors, which can result in a proinflammatory or immunomodulatory outcome. Assays are run with and without co-TNFa stimulation, in order to check for costimulatory or inhibitory activity.

[1731] Briefly, on day 1, 96-well black plates are set up with 1000 cells/well (NHDF) or 2000 cells/well (AoSMC) in 100 µl culture media. NHDF culture media contains: Clonetics FB basal media, 1mg/ml hFGF, 5mg/ml insulin, 50mg/ml gentamycin, 2%FBS, while AoSMC culture media contains Clonetics SM basal media, 0.5 µg/ml hEGF, 5mg/ml insulin, 1µg/ml hFGF, 50mg/ml gentamycin, 50 µg/ml Amphotericin B, 5%FBS. After incubation @ 37°C for at least 4-5 hours culture media is aspirated and replaced with growth arrest media. Growth arrest media for NHDF contains fibroblast basal media, 50mg/ml gentamycin, 2% FBS, while growth arrest media for AoSMC contains SM basal media, 50mg/ml gentamycin, 50µg/ml Amphotericin B, 0.4% FBS. Incubate at 37C until day 2.

[1732] On day 2, serial dilutions and templates of the polypeptide of interest are designed which should always include media controls and known-protein controls. For both stimulation and inhibition experiments, proteins are diluted in growth arrest media. For inhibition experiments, TNFa is added to a final concentration of 2ng/ml (NHDF) or 5ng/ml (AoSMC). Then add 1/3 vol media containing controls or supernatants and incubate at 37C/5% CO_2 until day 5.

[1733] Transfer 60µl from each well to another labeled 96-well plate, cover with a plate-sealer, and store at 4C until Day 6 (for IL6 ELISA). To the remaining 100 µl in the cell culture plate, aseptically add Alamar Blue in an amount equal to 10% of the culture volume (10µl). Return plates to incubator for 3 to 4 hours. Then measure fluorescence with excitation at 530nm and emission at 590nm using the CytoFluor. This yields the growth stimulation/inhibition data.

[1734] On day 5, the IL6 ELISA is performed by coating a 96 well plate with 50-100 ul/well of Anti-Human IL6 Monoclonal antibody diluted in PBS, pH 7.4, incubate ON at room temperature.

[1735] On day 6, empty the plates into the sink and blot on paper towels. Prepare Assay Buffer containing PBS with 4% BSA. Block the plates with 200 µl/well of Pierce Super Block blocking buffer in PBS for 1-2 hr and then wash plates with wash buffer (PBS, 0.05% Tween-20). Blot plates on paper towels. Then add 50 µl/well of diluted Anti-Human IL-6 Monoclonal, Biotin-labeled antibody at 0.50 mg/ml. Make dilutions of IL-6 stock in media (30, 10, 3, 1, 0.3, 0 ng/ml). Add duplicate samples to top row of plate. Cover the plates and incubate for 2 hours at RT on shaker.

[1736] Wash plates with wash buffer and blot on paper towels. Dilute EU-labeled Streptavidin 1:1000 in Assay buffer, and add 100 µl/well. Cover the plate and incubate 1 h at RT. Wash plates with wash buffer. Blot on paper towels.

[1737] Add 100 µl/well of Enhancement Solution. Shake for 5 minutes. Read the plate on the Wallac DELFIA Fluorometer. Readings from triplicate samples in each assay were tabulated and averaged.

[1738] A positive result in this assay suggests AoSMC cell proliferation and that the gene product of interest may be involved in dermal fibroblast proliferation and/or smooth muscle cell proliferation. A positive result also suggests many potential uses of polypeptides, polynucleotides, agonists and/or antagonists of the gene/gene product of interest. For example, inflammation and immune responses, wound healing, and angiogenesis, as detailed throughout this specification. Particularly, polypeptides of the gene product and polynucleotides of the gene may be used in wound healing and dermal regeneration, as well as the promotion of vasculargenesis, both of the blood vessels and lymphatics. The growth of vessels can be used in the treatment of, for example, cardiovascular diseases. Additionally, antagonists of polypeptides of the gene product and polynucleotides of the gene may be useful in treating diseases, disorders, and/or conditions which involve angiogenesis by acting as an anti-vascular (e.g., anti-angiogenesis). These diseases, disorders, and/or conditions are known in the art and/or are described herein, such as, for example, malignancies, solid tumors, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas,

trachomas, and pyogenic granulomas; artheroscleric plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uvietis and Pterygia (abnormal blood vessel growth) of the eye; rheumatoid arthritis; psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; fibromuscular dysplasia; wound granulation; Crohn's disease; and atherosclerosis. Moreover, antagonists of polypeptides of the gene product and polynucleotides of the gene may be useful in treating anti-hyperproliferative diseases and/or anti-inflammatory known in the art and/or described herein.

[1739] One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), antibodies, agonists, and/or antagonists and fragments and variants thereof.

[1740] Example 56: Cellular Adhesion Molecule (CAM) Expression on Endothelial Cells

[1741] The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

[1742] Briefly, endothelial cells (e.g., Human Umbilical Vein Endothelial cells (HUVECs)) are grown in a standard 96 well plate to confluence, growth medium is removed from the cells and replaced with 100 μl of 199 Medium (10% fetal bovine serum (FBS)). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10 μl volumes). Plates are then incubated at 37°C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μl of 0.1% paraformaldehyde-PBS(with Ca++ and Mg++) is added to each well. Plates are held at 4°C for 30 min. Fixative is removed from the wells and wells are washed 1X with PBS(+Ca,Mg) + 0.5% BSA and drained. 10 μl of diluted primary antibody is added to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μg/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed three times with PBS(+Ca,Mg) + 0.5% BSA. 20 μl of diluted ExtrAvidin-Alkaline Phosphotase (1:5,000 dilution, refered to herein as the working dilution) are added to each well and incubated at 37°C for 30 min. Wells are washed three times with PBS(+Ca,Mg)+0.5% BSA. Dissolve 1 tablet of p-Nitrophenol Phosphate pNPP per 5 ml of glycine buffer (pH 10.4). 100 μl of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphotase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μl of pNNP reagent is then added to each of the standard wells. The plate is incubated at 37°C for 4h. A volume of 50 μl of 3M NaOH is added to all wells. The plate is read on a plate reader at 405 nm using the background subtraction option on blank wells filled with glycine buffer only. Additionally, the template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

[1743] **Example 57: Alamar Blue Endothelial Cells Proliferation Assay**

[1744] This assay may be used to quantitatively determine protein mediated inhibition of bFGF-induced proliferation of Bovine Lymphatic Endothelial Cells (LECs), Bovine Aortic Endothelial Cells (BAECs) or Human Microvascular Uterine Myometrial Cells (UTMECs). This assay incorporates a fluorometric growth indicator based on detection of metabolic activity. A standard Alamar Blue Proliferation Assay is prepared in EGM-2MV with 10 ng /ml of bFGF added as a source of endothelial cell stimulation. This assay may be used with a variety of endothelial cells with slight changes in growth medium and cell concentration. Dilutions of the protein batches to be tested are diluted as appropriate. Serum-free medium (GIBCO SFM) without bFGF is used as a non-stimulated control and Angiostatin or TSP-1 are included as a known inhibitory controls.

[1745] Briefly, LEC, BAECs or UTMECs are seeded in growth media at a density of 5000 to 2000 cells/well in a 96 well plate and placed at 37-C overnight. After the overnight incubation of the cells, the growth media is removed and replaced with GIBCO EC-SFM. The cells are treated with the appropriate dilutions of the protein of interest or control protein sample(s) (prepared in SFM) in triplicate wells with additional bFGF to a concentration of 10 ng/ ml. Once the cells have been treated with the samples, the plate(s) is/are placed back in the 37° C incubator for three days. After three days 10 ml of stock alamar blue (Biosource Cat# DAL1100) is added to each well and the plate(s) is/are placed back in the 37°C incubator for four hours. The plate(s) are then read at 530nm excitation and 590nm emission using the CytoFluor fluorescence reader. Direct output is recorded in relative fluorescence units.

[1746] Alamar blue is an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. As cells grow in culture, innate metabolic activity results in a chemical reduction of the immediate surrounding environment. Reduction related to growth causes the indicator to change from oxidized (non-fluorescent blue) form to reduced (fluorescent red) form. i.e. stimulated proliferation will produce a stronger signal and inhibited proliferation will produce a weaker signal and the total signal is proportional to the total number of cells as well as their metabolic activity. The background level

of activity is observed with the starvation medium alone. This is compared to the output observed from the positive control samples (bFGF in growth medium) and protein dilutions.

[1747] Example 58: Detection of Inhibition of a Mixed Lymphocyte Reaction

[1748] This assay can be used to detect and evaluate inhibition of a Mixed Lymphocyte Reaction (MLR) by gene products (e.g., isolated polypeptides). Inhibition of a MLR may be due to a direct effect on cell proliferation and viability, modulation of costimulatory molecules on interacting cells, modulation of adhesiveness between lymphocytes and accessory cells, or modulation of cytokine production by accessory cells. Multiple cells may be targeted by these polypeptides since the peripheral blood mononuclear fraction used in this assay includes T, B and natural killer lymphocytes, as well as monocytes and dendritic cells.

[1749] Polypeptides of interest found to inhibit the MLR may find application in diseases associated with lymphocyte and monocyte activation or proliferation. These include, but are not limited to, diseases such as asthma, arthritis, diabetes, inflammatory skin conditions, psoriasis, eczema, systemic lupus erythematosus, multiple sclerosis, glomerulonephritis, inflammatory bowel disease, crohn's disease, ulcerative colitis, arteriosclerosis, cirrhosis, graft vs. host disease, host vs. graft disease, hepatitis, leukemia and lymphoma.

[1750] Briefly, PBMCs from human donors are purified by density gradient centrifugation using Lymphocyte Separation Medium (LSM®, density 1.0770 g/ml, Organon Teknika Corporation, West Chester, PA). PBMCs from two donors are adjusted to 2 x 10^6 cells/ml in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS and 2 mM glutamine. PBMCs from a third donor is adjusted to 2 x 10^5 cells/ml. Fifty microliters of PBMCs from each donor is added to wells of a 96-well round bottom microtiter plate. Dilutions of test materials (50 µl) is added in triplicate to microtiter wells. Test samples (of the protein of interest) are added for final dilution of 1:4; rhuIL-2 (R&D Systems, Minneapolis, MN, catalog number 202-IL) is added to a final concentration of 1 µg/ml; anti-CD4 mAb (R&D Systems, clone 34930.11, catalog number MAB379) is added to a final concentration

of 10 μg/ml. Cells are cultured for 7-8 days at 37°C in 5% CO_2, and 1 μC of [^{3}H] thymidine is added to wells for the last 16 hrs of culture. Cells are harvested and thymidine incorporation determined using a Packard TopCount. Data is expressed as the mean and standard deviation of triplicate determinations.

[1751] Samples of the protein of interest are screened in separate experiments and compared to the negative control treatment, anti-CD4 mAb, which inhibits proliferation of lymphocytes and the positive control treatment, IL-2 (either as recombinant material or supernatant), which enhances proliferation of lymphocytes.

[1752] One skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides (e.g., gene therapy), antibodies, agonists, and/or antagonists and fragments and variants thereof.

Table 7

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Met	1	.	.	B	.	.	.	.	0.24	0.41	.	.	.	-0.40	0.65
Ile	2	.	.	B	.	.	T	.	0.60	0.39	.	.	.	0.10	0.88
Pro	3	.	.	B	.	.	T	.	0.99	0.46	.	.	.	-0.20	0.93
Asn	4	.	.	.	.	T	T	.	0.79	0.43	.	.	.	0.35	1.51
Gln	5	.	.	B	.	.	T	.	0.83	0.31	.	.	F	0.40	2.18
His	6	.	.	.	.	.	.	C	0.84	0.06	.	.	F	0.40	1.40
Asn	7	.	.	.	.	.	.	C	1.39	0.13	.	.	.	0.10	0.88
Ala	8	.	.	.	.	.	.	C	1.30	0.16	.	.	.	0.10	0.50
Gly	9	.	.	.	.	T	T	.	1.27	0.14	.	.	.	0.50	0.49
Ala	10	.	.	.	.	T	T	.	1.27	0.14	.	.	F	0.65	0.42
Gly	11	.	.	.	.	.	T	C	1.09	0.14	.	.	F	0.45	0.72
Ser	12	.	.	.	.	.	T	C	0.50	0.07	.	.	F	0.60	1.12
His	13	.	A	.	.	.	.	C	0.23	0.14	.	.	F	0.20	1.12
Gln	14	.	A	B	.	.	.	.	-0.12	0.29	*	*	F	-0.15	0.84
Pro	15	.	A	B	.	.	.	.	0.58	0.64	*	*	F	-0.45	0.54
Ala	16	.	A	B	.	.	.	.	0.32	0.26	*	*	.	-0.30	0.78
Val	17	A	A	.	.	.	.	.	0.03	0.37	.	*	.	-0.30	0.45
Phe	18	.	A	B	.	.	.	.	-0.79	0.47	.	*	.	-0.60	0.29
Arg	19	.	A	B	.	.	.	.	-1.60	0.69	.	.	.	-0.60	0.21
Met	20	.	A	B	.	.	.	.	-1.39	0.87	.	*	.	-0.60	0.24
Ala	21	A	A	.	.	.	.	.	-1.11	0.23	.	*	.	-0.30	0.46
Val	22	A	A	.	.	.	.	.	-0.26	-0.07	.	*	.	0.30	0.34
Leu	23	A	A	.	.	.	.	.	-0.37	-0.07	.	*	.	0.30	0.57
Asp	24	A	.	.	.	.	T	.	-0.48	-0.00	.	*	F	0.85	0.47
Thr	25	A	.	.	.	.	T	.	0.09	-0.50	*	.	F	1.30	1.05
Asp	26	A	.	.	.	.	T	.	-0.21	-0.64	*	*	F	1.30	1.73
Leu	27	A	.	.	.	.	T	.	-0.17	-0.64	*	*	.	1.00	0.73
Asp	28	A	.	.	.	.	.	.	0.43	0.04	*	*	.	-0.10	0.41
His	29	.	.	B	.	.	.	.	0.13	-0.01	.	*	.	0.50	0.38
Ile	30	.	.	B	.	.	.	.	0.14	0.37	*	.	.	-0.10	0.62
Leu	31	.	.	B	.	.	T	.	-0.71	0.07	.	.	.	0.10	0.50
Pro	32	.	.	B	.	.	T	.	-0.71	0.71	.	.	F	-0.05	0.27
Ser	33	.	.	.	.	T	T	.	-0.92	0.90	.	.	F	0.35	0.32
Ser	34	.	.	B	.	.	T	.	-1.10	0.64	.	.	F	-0.05	0.60
Val	35	.	.	B	.	.	.	.	-0.91	0.39	.	.	F	0.05	0.60
Leu	36	.	.	B	.	.	.	.	-0.39	0.74	.	.	F	-0.25	0.39
Pro	37	.	.	B	.	.	T	.	-0.77	1.27	.	.	F	-0.05	0.31
Pro	38	.	.	B	.	.	T	.	-0.42	1.39	*	*	.	-0.20	0.42
Phe	39	A	.	.	.	.	T	.	-0.93	0.74	*	.	.	-0.05	1.01
Trp	40	A	.	.	.	.	T	.	-0.93	0.74	.	*	.	-0.20	0.54
Ala	41	A	A	.	B	.	.	.	-0.98	0.96	*	.	.	-0.60	0.26
Lys	42	.	A	B	B	.	.	.	-1.11	1.17	.	*	.	-0.60	0.22
Leu	43	.	A	B	B	.	.	.	-1.20	0.81	.	.	.	-0.60	0.21
Val	44	.	A	B	B	.	.	.	-1.36	0.29	.	.	.	-0.30	0.28
Val	45	.	.	B	B	.	.	.	-1.66	0.43	.	.	.	-0.60	0.10
Gly	46	.	.	B	B	.	.	.	-1.96	0.93	.	.	.	-0.60	0.13
Ser	47	.	A	B	B	.	.	.	-2.86	0.93	.	.	.	-0.60	0.12
Val	48	.	A	B	B	.	.	.	-2.71	0.93	.	.	.	-0.60	0.12
Ala	49	.	A	B	B	.	.	.	-2.56	0.86	.	.	.	-0.60	0.06
Ile	50	.	A	B	B	.	.	.	-2.29	1.21	*	.	.	-0.60	0.04
Val	51	.	A	B	B	.	.	.	-1.83	1.33	*	.	.	-0.60	0.06
Cys	52	.	A	B	B	.	.	.	-1.83	0.69	*	.	.	-0.60	0.11
Phe	53	.	A	B	B	.	.	.	-1.22	0.57	*	.	.	-0.26	0.21
Ala	54	.	A	B	B	.	.	.	-0.63	0.64	*	.	.	0.08	0.44
Arg	55	.	A	B	B	.	.	.	-0.09	-0.00	*	.	.	1.47	1.38
Ser	56	.	.	.	.	T	T	.	0.77	-0.14	*	.	F	2.76	1.57
Tyr	57	.	.	.	.	T	T	.	0.73	-0.93	.	.	F	3.40	2.60

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Asp	58	.	.	.	.	T	T	.	0.58	-0.64	.	.	F	3.06	1.15
Gly	59	.	.	.	.	T	T	.	0.47	-0.00	.	*	F	2.27	0.64
Asp	60	.	.	B	.	.	.	.	0.36	0.40	.	*	F	0.73	0.35
Phe	61	.	.	B	.	.	.	.	0.66	-0.36	.	*	.	0.84	0.35
Val	62	.	.	B	.	.	.	.	0.60	-0.36	.	*	.	0.50	0.59
Phe	63	.	.	B	.	.	.	.	0.60	-0.40	.	*	.	0.50	0.48
Asp	64	A	.	.	.	.	T	.	0.36	-0.40	*	.	F	0.85	0.95
Asp	65	A	.	.	.	.	T	.	-0.53	-0.69	*	.	F	1.30	1.30
Ser	66	A	.	.	.	.	T	.	-0.69	-0.64	*	.	F	1.30	1.05
Glu	67	A	.	.	.	.	T	.	0.17	-0.79	*	.	F	1.15	0.47
Ala	68	A	.	.	.	.	.	.	0.87	-0.39	.	.	.	0.50	0.45
Ile	69	A	.	.	.	.	.	.	0.91	0.01	.	.	.	-0.10	0.54
Val	70	A	.	.	.	.	.	.	0.91	-0.37	.	.	.	0.50	0.62
Asn	71	A	.	.	.	.	.	.	0.40	-0.37	.	.	F	0.80	1.03
Asn	72	A	.	.	.	.	T	.	0.40	-0.19	.	.	F	1.00	1.21
Lys	73	A	.	.	.	.	T	.	0.40	-0.47	.	*	F	1.00	2.83
Asp	74	A	.	.	.	.	T	C	1.29	-0.61	.	*	F	1.50	1.78
Leu	75	A	.	.	.	.	T	.	1.83	-1.01	.	.	F	1.30	1.91
Gln	76	.	.	B	.	.	.	.	1.62	-0.93	.	*	.	0.95	1.38
Ala	77	.	.	B	.	.	.	.	0.81	-0.50	.	*	F	1.35	1.28
Glu	78	.	.	B	.	.	.	.	0.42	0.19	.	*	F	0.70	1.28
Thr	79	.	.	B	.	.	T	.	0.42	-0.07	.	*	F	1.60	0.73
Pro	80	A	.	.	.	.	T	.	0.42	-0.47	*	*	F	2.00	1.21
Leu	81	.	.	.	.	T	T	.	0.13	-0.29	*	.	F	2.50	0.57
Gly	82	A	.	.	.	.	T	.	0.69	0.63	*	.	F	0.95	0.42
Asp	83	A	A	.	.	.	.	.	0.66	0.64	.	.	.	0.15	0.37
Leu	84	A	A	.	.	.	.	.	0.97	0.71	*	.	.	-0.10	0.61
Trp	85	A	A	.	.	.	.	.	0.48	0.03	.	.	.	0.10	1.03
His	86	.	A	B	.	.	.	.	1.00	0.39	.	.	.	-0.30	0.53
His	87	.	A	B	.	.	.	.	1.00	1.30	.	.	.	-0.60	0.68
Asp	88	.	A	.	.	T	.	.	0.70	1.04	.	.	.	-0.20	0.64
Phe	89	.	A	.	.	T	.	.	1.62	0.51	.	.	.	-0.20	0.63
Trp	90	.	A	.	.	T	.	.	1.10	0.01	.	*	.	0.10	0.91
Gly	91	.	.	.	.	.	T	C	0.83	0.20	.	.	F	0.45	0.45
Ser	92	.	.	.	.	.	T	C	0.57	0.59	.	*	F	0.15	0.69
Arg	93	.	.	.	.	.	T	C	0.57	0.19	.	*	F	0.45	0.88
Leu	94	.	.	.	.	.	T	C	0.96	-0.33	.	*	F	1.20	1.43
Ser	95	.	.	.	.	.	T	C	0.94	-0.27	*	*	F	1.20	1.54
Ser	96	.	.	.	.	.	T	C	1.26	-0.27	*	*	F	1.20	1.06
Asn	97	.	.	.	.	.	T	C	1.60	0.23	*	*	F	0.60	1.74
Thr	98	.	.	.	.	T	T	.	1.19	-0.46	*	*	F	1.74	2.60
Ser	99	.	.	.	.	T	.	.	1.76	-0.46	*	*	F	1.88	2.60
His	100	.	.	.	.	T	T	.	2.17	-0.09	*	.	F	2.42	2.53
Lys	101	.	.	.	.	T	T	.	2.26	-0.49	*	.	F	2.76	3.44
Ser	102	.	.	.	.	T	T	.	1.44	-0.54	*	.	F	3.40	3.97
Tyr	103	.	.	B	.	.	T	.	1.44	-0.24	*	.	F	2.36	2.40
Arg	104	.	.	B	B	.	.	.	0.89	-0.26	*	.	F	1.62	1.74
Pro	105	.	.	B	B	.	.	.	0.11	0.39	*	.	F	0.53	0.96
Leu	106	.	.	B	B	.	.	.	-0.24	0.69	*	.	.	-0.26	0.51
Thr	107	.	.	B	B	.	.	.	-0.64	0.41	*	*	.	-0.60	0.37
Val	108	.	.	B	B	.	.	.	-0.29	1.20	*	*	.	-0.60	0.21
Leu	109	.	.	B	B	.	.	.	-1.29	0.77	*	*	.	-0.60	0.50
Thr	110	.	.	B	B	.	.	.	-1.08	0.77	.	*	.	-0.60	0.24
Phe	111	.	.	B	B	.	.	.	-0.51	0.69	.	*	.	-0.60	0.52
Arg	112	.	.	B	B	.	.	.	-0.44	0.80	.	*	.	-0.60	0.99
Ile	113	.	.	B	B	.	.	.	-0.40	0.87	.	*	.	-0.45	1.08
Asn	114	.	.	B	B	.	.	.	0.11	1.07	.	*	.	-0.45	1.02
Tyr	115	.	.	B	B	.	.	.	0.08	0.67	.	*	.	-0.60	0.70

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Tyr	116	.	.	.	B	T	.	.	0.43	1.10	*	*	.	-0.20	0.99
Leu	117	.	.	.	.	T	T	.	-0.38	0.84	*	*	.	0.20	0.61
Ser	118	.	.	.	.	T	T	.	0.48	1.23	*	*	F	0.35	0.34
Gly	119	.	.	.	.	T	T	.	0.27	0.97	.	*	F	0.35	0.29
Gly	120	.	.	.	.	T	T	.	-0.34	0.64	.	.	F	0.35	0.55
Phe	121	.	.	B	.	.	.	.	-0.44	0.60	.	.	.	-0.40	0.30
His	122	.	.	B	.	.	T	.	-0.33	0.64	.	.	.	-0.20	0.30
Pro	123	.	.	B	.	.	T	.	-0.07	1.00	.	.	.	-0.20	0.27
Val	124	.	.	B	.	.	T	.	-0.58	1.07	.	.	.	-0.20	0.42
Gly	125	.	.	B	.	.	T	.	-1.09	0.93	*	.	.	-0.20	0.23
Phe	126	.	.	B	B	.	.	.	-0.39	1.07	*	.	.	-0.60	0.11
His	127	.	.	B	B	.	.	.	-1.24	1.04	*	.	.	-0.60	0.24
Val	128	.	.	B	B	.	.	.	-1.84	1.09	*	.	.	-0.60	0.17
Val	129	.	.	B	B	.	.	.	-1.80	1.34	*	.	.	-0.60	0.16
Asn	130	.	.	B	B	.	.	.	-1.49	1.24	*	.	.	-0.60	0.10
Ile	131	.	.	B	B	.	.	.	-1.09	1.24	*	.	.	-0.60	0.18
Leu	132	.	.	B	B	.	.	.	-1.40	0.99	*	*	.	-0.60	0.32
Leu	133	A	.	.	B	.	.	.	-1.43	0.77	.	*	.	-0.60	0.20
His	134	.	.	.	.	.	T	C	-0.88	1.06	*	*	.	0.00	0.20
Ser	135	.	.	.	.	.	T	C	-1.73	0.76	.	*	.	0.00	0.32
Gly	136	.	.	B	.	.	T	.	-1.66	0.71	.	*	.	-0.20	0.29
Ile	137	.	.	B	.	.	T	.	-1.44	0.71	.	.	.	-0.20	0.17
Ser	138	.	.	B	B	.	.	.	-1.49	0.83	.	.	.	-0.60	0.13
Val	139	.	.	B	B	.	.	.	-1.46	1.09	.	.	.	-0.60	0.10
Leu	140	.	.	B	B	.	.	.	-2.01	0.66	.	.	.	-0.60	0.23
Met	141	.	.	B	B	.	.	.	-2.37	0.61	.	.	.	-0.60	0.13
Val	142	.	.	B	B	.	.	.	-1.78	1.01	.	.	.	-0.60	0.15
Asp	143	.	.	B	B	.	.	.	-2.33	0.76	.	.	.	-0.60	0.24
Val	144	.	.	B	B	.	.	.	-2.29	0.71	.	.	.	-0.60	0.18
Phe	145	.	.	B	B	.	.	.	-2.18	0.79	.	.	.	-0.60	0.20
Ser	146	.	.	B	B	.	.	.	-1.92	0.93	*	.	.	-0.60	0.10
Val	147	.	.	B	B	.	.	.	-1.41	1.36	*	.	.	-0.60	0.14
Leu	148	.	.	B	B	.	.	.	-2.22	1.14	*	.	.	-0.60	0.16
Phe	149	.	.	B	B	.	.	.	-1.37	1.04	*	.	.	-0.60	0.10
Gly	150	.	.	.	B	T	.	.	-0.91	1.06	.	.	.	-0.20	0.23
Gly	151	.	.	.	B	.	.	C	-0.92	1.17	.	.	.	-0.40	0.44
Leu	152	.	.	B	B	.	.	.	-0.37	0.97	*	*	.	-0.60	0.73
Gln	153	.	.	B	B	.	.	.	0.49	0.57	*	*	.	-0.26	0.98
Tyr	154	.	.	B	B	.	.	.	0.84	0.14	.	*	F	0.68	1.98
Thr	155	.	.	.	B	T	.	.	1.30	0.14	*	*	F	1.42	2.38
Ser	156	.	.	.	.	T	T	.	1.76	-0.54	*	*	F	3.06	2.69
Lys	157	.	.	.	.	T	T	.	1.76	-0.94	*	*	F	3.40	3.37
Gly	158	.	.	.	.	T	T	.	1.72	-1.01	*	*	F	3.06	1.92
Arg	159	.	.	B	.	.	T	.	1.16	-1.00	*	.	F	2.32	1.95
Arg	160	.	A	B	.	.	.	.	0.88	-0.70	*	.	F	1.43	0.81
Leu	161	.	A	B	.	.	.	.	0.97	-0.20	*	*	.	0.64	0.82
His	162	.	A	B	.	.	.	.	1.03	-0.20	*	*	.	0.30	0.65
Leu	163	.	A	B	.	.	.	.	0.79	-0.20	*	*	.	0.30	0.65
Ala	164	.	A	B	.	.	.	.	0.38	0.30	*	*	.	-0.30	0.80
Pro	165	A	A	.	.	.	.	.	-0.54	0.00	.	*	F	-0.15	0.78
Arg	166	A	A	.	.	.	.	.	-0.54	0.19	*	*	F	-0.15	0.78
Ala	167	A	A	.	.	.	.	.	-1.10	0.19	.	*	.	-0.30	0.64
Ser	168	A	A	.	.	.	.	.	-0.88	0.19	.	*	.	-0.30	0.42
Leu	169	A	A	.	.	.	.	.	-1.10	0.26	.	*	.	-0.30	0.22
Leu	170	A	A	.	.	.	.	.	-1.70	0.94	.	*	.	-0.60	0.18
Ala	171	A	A	.	.	.	.	.	-2.51	1.13	.	*	.	-0.60	0.11
Ala	172	A	A	.	B	.	.	.	-2.51	1.53	.	.	.	-0.60	0.11

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Leu	173	A	A	.	B	.	.	.	-3.07	1.34	.	.	.	-0.60	0.14
Leu	174	A	A	.	B	.	.	.	-2.29	1.30	.	.	.	-0.60	0.10
Phe	175	A	A	.	B	.	.	.	-1.69	1.30	.	.	.	-0.60	0.14
Ala	176	A	A	.	B	.	.	.	-1.96	1.23	.	.	.	-0.60	0.26
Val	177	A	A	.	B	.	.	.	-1.40	1.19	.	.	.	-0.60	0.23
His	178	A	A	.	B	.	.	.	-0.90	1.00	.	.	.	-0.60	0.37
Pro	179	A	A	.	B	.	.	.	-0.09	0.70	.	.	.	-0.60	0.52
Val	180	A	A	.	B	.	.	.	-0.06	0.20	.	.	.	-0.15	1.22
His	181	A	A	.	B	.	.	.	-0.32	0.13	.	.	.	-0.30	0.48
Thr	182	A	A	.	B	.	.	.	-0.06	0.27	.	.	.	-0.30	0.23
Glu	183	A	A	B	B	.	.	.	-0.37	0.34	.	.	.	-0.30	0.31
Cys	184	.	A	B	B	.	.	.	-1.01	0.13	.	.	.	-0.30	0.23
Val	185	.	.	B	B	.	.	.	-1.01	0.27	.	.	.	-0.30	0.12
Ala	186	.	.	B	B	.	.	.	-1.32	0.43	*	.	.	-0.60	0.05
Gly	187	.	.	B	B	.	.	.	-0.90	0.86	*	*	.	-0.60	0.09
Val	188	.	.	B	B	.	.	.	-1.49	0.29	*	*	.	-0.30	0.24
Val	189	.	.	B	B	.	.	.	-0.82	0.14	.	*	.	-0.30	0.24
Gly	190	A	A	.	.	.	.	.	-0.78	-0.36	.	*	.	0.30	0.41
Arg	191	A	A	.	.	.	.	.	-1.00	-0.10	.	*	.	0.30	0.46
Ala	192	A	A	.	.	.	.	.	-1.32	-0.06	.	*	.	0.30	0.51
Asp	193	A	A	.	.	.	.	.	-1.06	-0.13	.	*	.	0.30	0.28
Leu	194	A	A	.	.	.	.	.	-1.01	-0.06	.	*	.	0.30	0.14
Leu	195	A	A	.	.	.	.	.	-1.37	0.63	.	*	.	-0.60	0.12
Cys	196	A	A	.	.	.	.	.	-2.18	0.91	.	*	.	-0.60	0.06
Ala	197	A	A	.	.	.	.	.	-2.40	1.70	.	.	.	-0.60	0.06
Leu	198	A	A	.	.	.	.	.	-3.21	1.70	*	.	.	-0.60	0.06
Phe	199	A	A	.	.	.	.	.	-2.70	1.70	.	.	.	-0.60	0.10
Phe	200	A	A	.	.	.	.	.	-2.59	1.51	.	.	.	-0.60	0.13
Leu	201	A	A	.	.	.	.	.	-2.73	1.80	.	.	.	-0.60	0.14
Leu	202	A	A	.	.	.	.	.	-2.49	1.80	.	.	.	-0.60	0.13
Ser	203	A	.	.	.	.	.	.	-1.92	1.44	.	.	.	-0.40	0.15
Phe	204	A	.	.	.	.	.	.	-1.89	1.41	.	.	.	-0.40	0.28
Leu	205	A	.	.	.	.	T	.	-1.14	1.30	.	*	.	-0.20	0.18
Gly	206	.	.	.	.	T	T	.	-0.92	0.61	.	.	.	0.20	0.27
Tyr	207	.	.	.	.	T	T	.	-0.81	0.73	*	*	.	0.20	0.32
Cys	208	A	.	.	.	.	T	.	-0.40	0.73	*	.	.	-0.20	0.33
Lys	209	A	A	.	.	.	.	.	0.30	0.04	*	.	.	-0.30	0.66
Ala	210	A	A	.	.	.	.	.	0.81	-0.39	*	.	.	0.30	0.73
Phe	211	A	A	.	.	.	.	.	1.16	-0.76	*	.	.	0.75	1.82
Arg	212	A	A	.	.	.	.	.	1.44	-0.93	*	.	F	0.90	1.47
Glu	213	A	A	.	.	.	.	.	2.11	-0.93	*	.	F	0.90	2.90
Ser	214	A	A	.	.	.	.	.	1.72	-1.43	*	.	F	0.90	5.80
Asn	215	A	.	.	.	.	T	.	1.72	-1.79	*	.	F	1.30	2.93
Lys	216	A	.	.	.	.	T	.	2.39	-1.29	*	*	F	1.30	1.71
Glu	217	A	.	.	.	.	T	.	1.98	-0.79	*	.	F	1.30	1.74
Gly	218	A	.	.	.	.	T	.	1.68	-0.79	.	.	F	1.30	1.45
Ala	219	A	.	.	.	.	.	.	1.67	-0.80	.	.	F	0.95	0.97
His	220	.	.	.	.	.	T	C	0.97	-0.31	.	.	F	1.05	0.81
Ser	221	.	.	.	.	.	T	C	0.63	0.47	.	.	F	0.15	0.71
Ser	222	.	.	.	.	.	T	C	-0.22	0.96	.	.	F	0.15	0.74
Thr	223	.	.	B	.	.	T	.	-0.69	1.10	.	.	F	-0.05	0.40
Phe	224	.	.	B	B	.	.	.	-0.91	1.29	.	.	.	-0.60	0.25
Trp	225	.	.	B	B	.	.	.	-1.18	1.59	.	.	.	-0.60	0.15
Val	226	.	.	B	B	.	.	.	-1.77	1.59	.	.	.	-0.60	0.14
Leu	227	.	.	B	B	.	.	.	-2.17	1.79	.	.	.	-0.60	0.11
Leu	228	.	.	B	B	.	.	.	-2.67	1.79	.	.	.	-0.60	0.09
Ser	229	.	.	B	B	.	.	.	-2.31	1.56	.	.	.	-0.60	0.10
Ile	230	A	.	.	B	.	.	.	-2.61	1.34	.	.	.	-0.60	0.13

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Phe	231	A	.	.	B	.	.	.	-2.61	1.16	.	.	.	-0.60	0.15
Leu	232	A	.	.	B	.	.	.	-2.39	1.11	.	.	.	-0.60	0.09
Gly	233	A	.	.	B	.	.	.	-2.18	1.23	.	.	.	-0.60	0.12
Ala	234	A	.	.	B	.	.	.	-2.69	1.16	.	.	.	-0.60	0.14
Val	235	A	.	.	B	.	.	.	-2.47	1.06	*	.	.	-0.60	0.14
Ala	236	A	.	.	B	.	.	.	-1.72	0.94	*	.	.	-0.60	0.08
Met	237	A	.	.	B	.	.	.	-0.91	0.51	.	.	.	-0.60	0.15
Leu	238	A	.	.	B	.	.	.	-0.57	0.01	.	.	.	-0.30	0.35
Cys	239	A	.	.	B	.	.	.	-0.32	-0.23	.	.	.	0.30	0.60
Lys	240	A	.	.	B	.	.	.	-0.36	-0.30	.	.	F	0.45	0.60
Glu	241	A	.	.	.	.	.	.	-0.08	-0.23	.	.	F	0.65	0.51
Gln	242	A	.	.	B	.	.	.	-0.33	-0.43	.	.	F	0.60	1.38
Gly	243	.	.	B	B	.	.	.	-0.33	-0.36	.	.	F	0.45	0.51
Ile	244	.	.	B	B	.	.	.	-0.01	0.33	.	.	F	-0.15	0.24
Thr	245	.	.	B	B	.	.	.	-0.87	0.76	.	.	.	-0.60	0.14
Val	246	.	.	B	B	.	.	.	-0.87	1.04	.	.	.	-0.60	0.12
Leu	247	.	.	B	B	.	.	.	-1.46	1.01	.	.	.	-0.60	0.27
Gly	248	.	.	B	B	.	.	.	-1.97	0.83	.	.	.	-0.60	0.19
Leu	249	.	.	B	B	.	.	.	-1.78	0.99	.	*	.	-0.60	0.19
Asn	250	.	.	B	B	.	.	.	-1.47	1.13	.	*	.	-0.60	0.20
Ala	251	A	.	.	B	.	.	.	-1.50	0.44	*	*	.	-0.60	0.33
Val	252	A	.	.	B	.	.	.	-1.50	0.70	*	*	.	-0.60	0.28
Phe	253	.	.	B	B	.	.	.	-2.01	0.70	*	*	.	-0.60	0.14
Asp	254	.	.	B	B	.	.	.	-2.09	0.94	*	*	.	-0.60	0.11
Ile	255	.	.	B	B	.	.	.	-2.43	1.13	*	*	.	-0.60	0.10
Leu	256	.	.	B	B	.	.	.	-1.80	0.91	.	*	.	-0.60	0.11
Val	257	.	.	B	B	.	.	.	-1.64	0.13	.	*	.	-0.30	0.14
Ile	258	A	.	.	B	.	.	.	-0.94	0.91	.	*	.	-0.60	0.17
Gly	259	A	.	.	B	.	.	.	-1.80	0.63	.	*	.	-0.60	0.33
Lys	260	A	.	.	B	.	.	.	-1.72	0.59	.	*	.	-0.60	0.33
Phe	261	A	.	.	B	.	.	.	-0.91	0.63	.	.	.	-0.60	0.39
Asn	262	A	.	.	B	.	.	.	-0.94	-0.06	.	*	.	0.30	0.68
Val	263	A	.	.	B	.	.	.	-0.44	0.20	.	.	.	-0.30	0.24
Leu	264	A	.	.	B	.	.	.	-0.10	0.63	.	*	.	-0.60	0.35
Glu	265	A	.	.	B	.	.	.	-0.10	0.24	*	*	.	-0.30	0.38
Ile	266	A	.	.	B	.	.	.	-0.26	-0.16	*	.	F	0.60	1.02
Xxx	267	A	.	.	B	.	.	.	-1.07	-0.16	*	.	F	0.45	0.91
Gln	268	A	.	.	B	.	.	.	-0.24	-0.16	*	*	F	0.45	0.44
Lys	269	A	.	.	B	.	.	.	0.61	0.34	*	*	F	-0.15	0.85
Val	270	A	.	.	B	.	.	.	0.61	-0.34	*	*	F	0.60	1.32
Leu	271	A	.	.	B	.	.	.	1.54	-0.77	*	*	.	0.75	1.28
His	272	A	.	.	B	.	.	.	1.63	-1.17	*	.	.	0.75	1.28
Lys	273	A	.	.	B	.	.	.	0.82	-0.79	*	.	F	0.90	2.30
Asp	274	A	.	.	.	.	T	.	0.78	-0.74	*	.	F	1.30	2.30
Lys	275	A	.	.	.	.	T	.	1.63	-1.43	*	.	F	1.30	2.93
Ser	276	A	.	.	.	.	T	.	1.63	-1.53	.	.	F	1.30	2.36
Leu	277	A	.	.	.	.	T	.	1.32	-0.84	.	.	F	1.30	1.16
Glu	278	A	A	.	.	.	.	.	0.68	-0.41	.	.	F	0.45	0.58
Asn	279	A	A	.	.	.	.	.	-0.13	0.20	.	.	.	-0.30	0.43
Leu	280	A	A	.	.	.	.	.	-0.07	0.50	.	.	.	-0.60	0.43
Gly	281	A	A	.	.	.	.	.	0.23	-0.19	.	.	.	0.30	0.48
Met	282	.	A	B	.	.	.	.	0.70	0.21	.	.	.	-0.30	0.48
Leu	283	.	.	B	.	.	T	.	0.36	0.24	.	.	.	0.10	0.58
Arg	284	.	.	B	.	.	T	.	-0.46	-0.01	.	.	F	0.85	0.58
Asn	285	.	.	.	.	T	T	.	-0.46	0.24	*	.	F	0.65	0.48
Gly	286	.	.	.	.	T	T	.	-0.81	0.31	*	*	F	0.65	0.48
Gly	287	.	.	.	B	.	.	C	-0.10	0.41	*	*	F	-0.25	0.21
Leu	288	.	.	B	B	.	.	.	0.11	0.41	*	*	.	-0.60	0.26

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Leu	289	.	.	B	B	.	.	.	-0.31	0.63	*	*	.	-0.60	0.26
Phe	290	.	.	B	B	.	.	.	-1.12	0.69	.	*	.	-0.60	0.38
Arg	291	.	.	B	B	.	.	.	-1.59	0.94	.	*	.	-0.60	0.38
Met	292	.	.	B	B	.	.	.	-1.56	0.94	.	*	.	-0.60	0.38
Thr	293	.	.	B	B	.	.	.	-1.04	0.74	.	*	.	-0.60	0.63
Leu	294	.	.	B	B	.	.	.	-0.58	0.34	.	*	.	-0.30	0.43
Leu	295	.	.	B	B	.	.	.	-0.22	0.77	.	*	F	-0.45	0.43
Thr	296	.	.	.	.	.	T	C	-0.92	0.59	.	*	F	0.15	0.30
Ser	297	.	.	.	.	.	T	C	-0.67	0.60	.	.	F	0.15	0.36
Gly	298	.	.	.	.	.	T	C	-0.96	0.34	.	.	F	0.45	0.43
Gly	299	.	.	.	.	.	T	C	-0.96	0.27	.	.	F	0.45	0.30
Ala	300	.	.	.	.	.	.	C	-0.39	0.47	.	.	F	-0.05	0.18
Gly	301	.	.	B	B	.	.	.	-0.93	0.84	.	*	.	-0.60	0.29
Met	302	.	.	B	B	.	.	.	-0.52	1.06	.	*	.	-0.60	0.22
Leu	303	.	.	B	B	.	.	.	-0.47	0.63	.	*	.	-0.60	0.42
Tyr	304	.	.	B	B	.	.	.	-0.01	1.04	.	*	.	-0.60	0.45
Val	305	.	.	B	B	.	.	.	-0.31	0.61	.	*	.	-0.60	0.89
Arg	306	.	.	B	B	.	.	.	-0.57	0.69	.	*	.	-0.60	0.75
Trp	307	.	.	B	B	.	.	.	-0.31	0.61	.	*	.	-0.60	0.48
Arg	308	.	.	B	B	.	.	.	0.19	0.29	.	*	.	-0.30	0.63
Ile	309	.	.	B	B	.	.	.	0.09	0.13	.	*	.	-0.30	0.47
Met	310	.	.	B	B	.	.	.	0.73	0.56	.	*	.	-0.60	0.44
Gly	311	.	.	.	.	T	.	.	0.23	0.07	.	*	.	0.30	0.35
Thr	312	.	.	.	.	.	.	C	-0.07	0.50	*	*	F	-0.05	0.63
Gly	313	.	.	.	.	.	T	C	-0.88	0.31	*	*	F	0.45	0.65
Pro	314	.	.	.	.	.	T	C	-0.30	0.49	*	.	F	0.15	0.57
Xxx	315	.	.	.	.	.	T	C	0.30	0.54	*	.	F	0.15	0.57
Ala	316	.	.	B	.	.	T	.	-0.21	0.06	.	.	.	0.10	0.99
Phe	317	.	.	B	.	.	.	.	0.10	0.27	*	.	.	-0.10	0.48
Thr	318	.	.	B	.	.	.	.	0.44	-0.16	*	.	.	0.77	0.62
Glu	319	.	.	B	.	.	.	.	0.44	-0.19	*	.	F	1.19	0.99
Val	320	.	.	B	.	.	.	.	0.24	-0.26	*	.	F	1.61	1.77
Asp	321	.	.	.	.	.	.	C	0.53	-0.54	*	*	F	2.38	1.24
Asn	322	.	.	.	.	.	T	C	0.53	-0.64	*	.	F	2.70	0.96
Pro	323	A	.	.	.	.	T	.	0.26	0.14	*	.	F	1.48	1.12
Ala	324	A	.	.	.	.	T	.	0.26	-0.00	*	.	F	1.66	0.68
Ser	325	A	.	.	.	.	T	.	0.81	-0.00	*	.	.	1.24	0.70
Phe	326	A	.	.	.	.	.	.	0.21	-0.01	*	.	.	0.77	0.61
Ala	327	A	.	.	.	.	.	.	-0.60	0.17	.	.	.	-0.10	0.60
Asp	328	A	.	.	.	.	.	.	-1.24	0.36	*	*	.	-0.10	0.37
Ser	329	A	.	.	B	.	.	.	-0.54	0.61	*	*	.	-0.60	0.31
Met	330	A	.	.	B	.	.	.	-0.83	-0.17	*	.	.	0.30	0.61
Leu	331	A	.	.	B	.	.	.	-0.99	-0.17	*	.	.	0.30	0.37
Val	332	.	.	B	B	.	.	.	-0.40	0.47	*	.	.	-0.60	0.20
Arg	333	.	.	B	B	.	.	.	-0.64	0.49	*	.	.	-0.60	0.33
Ala	334	.	.	B	B	.	.	.	-0.34	0.63	*	.	.	-0.60	0.63
Val	335	.	.	B	B	.	.	.	0.01	0.34	*	.	.	-0.15	1.36
Asn	336	.	.	B	.	.	T	.	0.58	0.46	*	.	.	-0.05	1.09
Tyr	337	.	.	B	.	.	T	.	1.19	1.21	*	.	.	-0.05	1.69
Asn	338	.	.	B	.	.	T	.	0.78	1.47	.	*	.	-0.05	3.57
Tyr	339	.	.	B	.	.	T	.	0.56	1.21	.	*	.	-0.05	2.98
Tyr	340	.	.	B	.	.	.	.	1.41	1.50	.	*	.	-0.25	1.57
Tyr	341	.	.	B	.	.	.	.	0.82	1.14	.	*	.	-0.25	1.57
Ser	342	.	A	B	.	.	.	.	0.78	1.24	.	*	.	-0.45	1.01
Leu	343	.	A	B	.	.	.	.	-0.03	1.40	.	*	.	-0.60	0.68
Asn	344	.	A	B	.	.	.	.	-0.60	1.33	.	*	.	-0.60	0.36
Ala	345	.	A	B	.	.	.	.	-1.17	1.26	.	.	.	-0.60	0.22

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI			XII	XII
Trp	346	.	A	B	.	.	.	.	-1.59	1.56	.	*	.	-0.60	0.22	
Leu	347	.	A	B	.	.	.	.	-1.50	1.44	.	.	.	-0.60	0.07	
Leu	348	.	A	B	.	.	.	.	-0.98	1.47	.	.	.	-0.60	0.11	
Leu	349	.	A	B	.	.	.	.	-1.27	1.89	.	.	.	-0.60	0.11	
Cys	350	.	.	B	.	.	T	.	-1.49	1.89	.	.	.	-0.20	0.14	
Pro	351	.	.	.	.	T	T	.	-1.87	1.89	.	.	.	0.20	0.14	
Trp	352	.	.	.	.	T	T	.	-1.76	1.77	.	.	.	0.20	0.09	
Trp	353	.	.	B	.	.	T	.	-0.94	1.87	.	*	.	-0.20	0.15	
Leu	354	.	.	B	.	.	.	.	-0.42	1.30	.	*	.	-0.40	0.16	
Cys	355	.	.	.	.	T	T	.	-0.06	1.79	.	*	.	0.20	0.16	
Phe	356	.	.	.	.	T	T	.	-0.44	1.26	.	*	.	0.20	0.21	
Asp	357	.	.	.	.	T	T	.	-0.50	0.96	.	*	.	0.20	0.25	
Trp	358	.	.	.	.	T	T	.	-0.88	0.70	.	*	.	0.20	0.46	
Ser	359	.	.	.	.	T	T	.	-0.96	0.70	.	*	.	0.20	0.28	
Met	360	.	.	.	.	T	T	.	-0.50	0.60	.	*	.	0.20	0.12	
Gly	361	.	.	.	.	T	T	.	-0.61	1.03	.	.	.	0.20	0.17	
Cys	362	.	.	B	.	.	T	.	-1.50	0.80	*	.	.	-0.20	0.11	
Ile	363	.	.	B	B	.	.	.	-1.17	1.10	.	.	.	-0.60	0.08	
Pro	364	.	.	B	B	.	.	.	-1.17	0.49	*	.	.	-0.60	0.15	
Leu	365	.	.	B	B	.	.	.	-1.46	0.44	*	.	.	-0.60	0.38	
Ile	366	.	.	B	B	.	.	.	-1.41	0.56	*	.	.	-0.60	0.38	
Lys	367	.	.	B	B	.	.	.	-0.74	0.26	*	.	F	-0.15	0.33	
Ser	368	.	.	B	B	.	.	.	-0.14	-0.17	*	*	F	0.45	0.68	
Ile	369	.	.	.	B	T	.	.	0.18	0.06	*	*	F	0.40	1.01	
Ser	370	.	.	B	B	.	.	.	0.13	-0.63	*	*	F	0.75	0.99	
Asp	371	.	.	.	B	T	.	.	0.13	0.01	.	*	F	0.25	0.55	
Trp	372	.	.	B	B	.	.	.	-0.50	0.31	.	*	.	-0.30	0.55	
Arg	373	.	A	B	.	.	.	.	-1.01	0.13	.	.	.	-0.30	0.41	
Val	374	.	A	B	.	.	.	.	-0.71	0.43	.	.	.	-0.60	0.20	
Ile	375	.	A	B	.	.	.	.	-1.00	0.93	.	*	.	-0.60	0.20	
Ala	376	A	A	.	.	.	.	.	-1.81	0.51	.	*	.	-0.60	0.10	
Leu	377	A	A	.	.	.	.	.	-1.81	1.20	*	*	.	-0.60	0.11	
Ala	378	A	A	.	.	.	.	.	-2.62	1.47	*	.	.	-0.60	0.17	
Ala	379	A	A	.	.	.	.	.	-2.43	1.57	.	.	.	-0.60	0.14	
Leu	380	A	A	.	.	.	.	.	-2.36	1.64	.	.	.	-0.60	0.09	
Trp	381	A	A	.	.	.	.	.	-2.66	1.64	.	.	.	-0.60	0.08	
Phe	382	A	A	.	.	.	.	.	-2.19	1.83	.	.	.	-0.60	0.05	
Cys	383	A	A	.	.	.	.	.	-2.41	1.76	.	.	.	-0.60	0.06	
Leu	384	A	A	.	.	.	.	.	-2.71	1.76	.	.	.	-0.60	0.05	
Ile	385	.	A	B	.	.	.	.	-2.57	1.53	*	.	.	-0.60	0.04	
Gly	386	.	A	.	.	T	.	.	-2.28	1.31	*	.	.	-0.20	0.04	
Leu	387	.	A	.	.	T	.	.	-2.17	1.14	*	.	.	-0.20	0.09	
Ile	388	.	A	B	.	.	.	.	-2.31	0.96	*	.	.	-0.60	0.12	
Cys	389	.	A	B	.	.	.	.	-2.17	0.96	*	.	.	-0.60	0.10	
Gln	390	.	A	B	.	.	.	.	-1.58	1.10	.	.	.	-0.60	0.07	
Ala	391	.	A	B	.	.	.	.	-1.23	0.80	.	.	.	-0.60	0.13	
Leu	392	.	A	B	.	.	.	.	-0.42	0.11	.	.	.	-0.30	0.41	
Cys	393	A	A	.	.	.	.	.	0.12	-0.46	.	.	.	0.30	0.40	
Ser	394	A	.	.	.	.	T	.	0.76	-0.43	.	.	F	0.85	0.39	
Glu	395	A	.	.	.	.	T	.	0.80	-0.43	.	.	F	0.85	0.64	
Asp	396	A	.	.	.	.	T	.	1.50	-1.11	.	.	F	1.30	2.39	
Gly	397	A	.	.	.	.	T	.	2.42	-1.69	.	.	F	1.30	3.50	
His	398	A	.	.	.	.	.	.	2.20	-2.07	.	.	F	1.10	3.95	
Lys	399	A	.	.	B	.	.	.	1.69	-1.39	.	.	F	0.90	1.66	
Arg	400	.	.	B	B	.	.	.	1.38	-0.70	.	.	F	0.90	1.38	
Arg	401	.	.	B	B	.	.	.	0.57	-0.64	*	.	.	0.75	1.47	
Ile	402	.	.	B	B	.	.	.	0.57	-0.46	*	.	.	0.30	0.61	
Leu	403	.	.	B	B	.	.	.	-0.21	-0.03	*	.	.	0.30	0.31	

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Thr	404	.	.	B	B	.	.	.	-0.60	0.66	*	.	.	-0.60	0.13
Leu	405	.	.	B	B	.	.	.	-1.41	1.09	*	.	.	-0.60	0.18
Gly	406	.	.	B	B	.	.	.	-2.33	1.19	*	*	.	-0.60	0.19
Leu	407	.	.	B	B	.	.	.	-2.30	1.19	.	.	.	-0.60	0.11
Gly	408	.	.	B	B	.	.	.	-2.38	1.34	.	.	.	-0.60	0.10
Phe	409	.	.	B	B	.	.	.	-2.28	1.34	.	.	.	-0.60	0.07
Leu	410	.	.	B	B	.	.	.	-2.17	1.34	.	.	.	-0.60	0.13
Val	411	.	.	B	B	.	.	.	-2.63	1.44	.	.	.	-0.60	0.11
Ile	412	.	.	B	B	.	.	.	-2.03	1.70	.	.	.	-0.60	0.11
Pro	413	.	.	B	.	.	.	.	-2.28	1.34	.	.	.	-0.40	0.20
Phe	414	.	.	B	.	.	.	.	-1.88	1.16	.	.	.	-0.40	0.28
Leu	415	.	.	B	.	.	.	.	-1.07	0.90	.	.	.	-0.40	0.53
Pro	416	.	.	.	.	.	.	C	-1.02	0.61	.	.	.	-0.20	0.55
Ala	417	.	.	.	.	T	T	.	-0.83	0.87	.	.	.	0.20	0.52
Ser	418	.	.	.	.	.	T	C	-1.32	0.87	*	*	.	0.00	0.55
Asn	419	A	.	.	.	.	T	.	-0.51	0.97	*	*	.	-0.20	0.31
Leu	420	.	.	B	.	.	T	.	-0.56	0.54	*	*	.	-0.20	0.60
Phe	421	.	.	B	B	.	.	.	-0.69	0.69	*	*	.	-0.60	0.33
Phe	422	.	.	B	B	.	.	.	-0.80	0.73	*	*	.	-0.60	0.20
Arg	423	.	.	B	B	.	.	.	-1.36	1.11	*	*	.	-0.60	0.21
Val	424	.	.	B	B	.	.	.	-2.21	1.07	*	*	.	-0.60	0.18
Gly	425	.	.	B	B	.	.	.	-1.99	0.93	*	*	.	-0.60	0.16
Phe	426	.	.	B	B	.	.	.	-1.29	0.64	*	*	.	-0.60	0.08
Val	427	A	.	.	B	.	.	.	-0.48	0.64	*	*	.	-0.60	0.19
Val	428	A	.	.	B	.	.	.	-1.44	-0.00	*	*	.	0.30	0.37
Ala	429	A	.	.	B	.	.	.	-1.40	0.21	*	.	.	-0.30	0.32
Glu	430	A	.	.	B	.	.	.	-1.30	0.11	*	.	.	-0.30	0.36
Arg	431	.	.	B	B	.	.	.	-1.41	0.23	*	.	.	-0.30	0.75
Val	432	.	.	B	B	.	.	.	-0.77	0.27	*	.	.	-0.30	0.62
Leu	433	.	.	B	B	.	.	.	-0.21	0.20	*	.	.	-0.30	0.55
Tyr	434	.	.	B	B	.	.	.	-0.01	0.59	*	.	.	-0.60	0.38
Leu	435	.	.	B	B	.	.	.	-0.36	1.01	*	.	.	-0.60	0.65
Pro	436	.	.	.	B	T	.	.	-0.71	0.80	*	.	F	-0.05	0.78
Ser	437	.	.	.	.	T	T	.	-0.52	0.87	.	.	F	0.35	0.78
Xxx	438	.	.	.	.	T	T	.	-0.57	0.69	.	.	F	0.35	0.50
Gly	439	.	.	B	.	.	T	.	-1.13	0.64	.	.	F	-0.05	0.24
Tyr	440	.	.	B	.	.	T	.	-1.13	0.90	.	.	.	-0.20	0.15
Cys	441	.	.	B	B	.	.	.	-1.23	1.20	.	.	.	-0.60	0.10
Val	442	.	.	B	B	.	.	.	-1.63	1.26	.	.	.	-0.60	0.14
Leu	443	.	.	B	B	.	.	.	-1.59	1.61	.	.	.	-0.60	0.08
Leu	444	.	.	B	B	.	.	.	-1.94	1.29	.	.	.	-0.60	0.14
Thr	445	.	.	B	B	.	.	.	-2.04	1.50	.	*	.	-0.60	0.17
Phe	446	.	.	B	B	.	.	.	-1.97	1.29	.	.	.	-0.60	0.20
Gly	447	A	.	.	B	.	.	.	-1.92	1.10	.	.	.	-0.60	0.25
Phe	448	A	.	.	B	.	.	.	-1.41	1.10	*	.	.	-0.60	0.14
Gly	449	A	.	.	.	.	.	.	-0.56	1.00	.	.	.	-0.40	0.22
Ala	450	A	.	.	.	.	.	.	-0.28	0.21	.	.	.	-0.10	0.44
Leu	451	A	.	.	.	.	.	.	0.11	0.29	*	.	.	-0.10	0.69
Ser	452	A	.	.	.	.	T	.	0.50	-0.01	*	.	F	1.00	1.01
Lys	453	A	.	.	.	.	T	.	1.24	-0.44	*	.	F	1.00	1.99
His	454	A	.	.	.	.	T	.	1.63	-0.94	.	.	F	1.30	4.83
Thr	455	A	.	.	.	.	T	.	2.27	-1.63	.	.	F	1.30	7.20
Lys	456	A	A	.	.	.	.	.	2.27	-2.01	.	.	F	0.90	7.20
Lys	457	A	A	.	.	.	.	.	1.68	-1.33	.	.	F	0.90	4.36
Lys	458	A	A	.	B	.	.	.	1.04	-1.14	.	.	F	0.90	2.12
Lys	459	A	A	.	B	.	.	.	0.49	-1.13	.	.	F	0.90	1.07
Leu	460	A	A	.	B	.	.	.	-0.06	-0.63	.	.	.	0.60	0.54
Ile	461	A	A	.	B	.	.	.	-0.96	0.01	.	.	.	-0.30	0.20

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Ala	462	A	A	.	B	.	.	.	-1.81	0.66	.	.	.	-0.60	0.07
Ala	463	A	A	.	B	.	.	.	-2.20	1.34	*	.	.	-0.60	0.07
Val	464	A	A	.	B	.	.	.	-3.13	1.09	*	.	.	-0.60	0.11
Val	465	.	A	B	B	.	.	.	-3.13	1.09	.	.	.	-0.60	0.07
Leu	466	.	A	B	B	.	.	.	-2.94	1.27	.	.	.	-0.60	0.06
Gly	467	.	A	B	B	.	.	.	-3.24	1.56	.	.	.	-0.60	0.07
Ile	468	.	.	B	B	.	.	.	-2.66	1.60	.	.	.	-0.60	0.07
Leu	469	.	.	B	B	.	.	.	-2.11	1.36	.	.	.	-0.60	0.13
Phe	470	.	.	B	B	.	.	.	-2.07	1.16	*	.	.	-0.60	0.19
Ile	471	.	.	B	B	.	.	.	-1.14	1.41	*	*	.	-0.60	0.22
Asn	472	.	.	B	B	.	.	.	-1.47	0.73	*	.	.	-0.60	0.52
Thr	473	.	.	B	B	.	.	.	-1.43	0.61	*	*	.	-0.60	0.32
Leu	474	.	.	B	B	.	.	.	-1.43	0.47	*	*	.	-0.60	0.34
Arg	475	.	.	B	B	.	.	.	-0.62	0.47	*	*	.	-0.60	0.18
Cys	476	.	.	B	B	.	.	.	-0.03	0.07	*	*	.	-0.30	0.24
Val	477	.	.	B	B	.	.	.	-0.38	-0.03	*	*	.	0.30	0.39
Leu	478	.	.	B	B	.	.	.	-0.07	-0.29	*	*	.	0.60	0.20
Arg	479	.	.	B	B	.	.	.	0.46	-0.29	*	*	F	1.05	0.63
Ser	480	.	.	.	.	.	T	C	0.46	0.06	*	*	F	1.35	0.89
Gly	481	.	.	.	.	.	T	C	0.82	-0.59	.	*	F	2.70	2.12
Glu	482	.	.	.	.	.	T	C	1.68	-0.89	*	*	F	3.00	1.45
Trp	483	.	.	.	.	.	T	C	2.49	-0.89	.	*	F	2.70	1.88
Arg	484	A	A	.	.	.	.	.	2.38	-1.27	.	*	F	1.80	3.29
Ser	485	A	A	.	.	.	.	.	1.87	-1.30	*	*	F	1.50	3.29
Glu	486	A	A	.	.	.	.	.	1.51	-0.61	*	*	F	1.20	2.58
Glu	487	A	A	.	.	.	.	.	1.62	-0.74	*	*	F	0.90	1.14
Gln	488	A	A	.	.	.	.	.	1.61	-0.74	*	*	F	0.90	1.67
Leu	489	A	A	.	.	.	.	.	0.91	-0.74	*	*	F	0.90	1.29
Phe	490	A	A	.	.	.	.	.	0.40	-0.24	*	.	.	0.30	0.75
Arg	491	A	A	.	.	.	.	.	0.10	0.44	*	*	.	-0.60	0.36
Ser	492	A	A	.	.	.	.	.	-0.76	0.43	*	*	.	-0.60	0.58
Ala	493	A	A	.	.	.	.	.	-1.42	0.39	*	*	.	-0.30	0.50
Leu	494	.	A	B	.	.	.	.	-0.82	0.17	*	*	.	-0.30	0.14
Ser	495	.	A	B	.	.	.	.	-0.93	0.60	.	*	.	-0.60	0.16
Val	496	.	.	B	.	.	.	.	-1.04	0.90	.	*	.	-0.40	0.13
Cys	497	.	.	B	.	.	T	.	-1.33	0.80	.	*	.	-0.20	0.25
Pro	498	A	.	.	.	.	T	.	-0.70	0.61	.	*	.	-0.20	0.19
Leu	499	A	.	.	.	.	T	.	-0.74	0.23	.	*	.	0.10	0.51
Asn	500	A	.	.	.	.	T	.	-0.48	0.23	.	*	.	0.10	0.70
Ala	501	A	.	.	.	.	.	.	0.13	0.16	.	*	.	-0.10	0.62
Lys	502	A	.	.	B	.	.	.	0.80	0.49	.	*	.	-0.45	1.18
Val	503	.	.	B	B	.	.	.	0.12	0.20	.	*	.	-0.15	1.18
His	504	.	.	B	B	.	.	.	0.59	0.49	*	*	.	-0.60	0.82
Tyr	505	.	.	B	B	.	.	.	0.63	0.41	*	*	.	-0.60	0.40
Asn	506	.	.	B	B	.	.	.	1.22	0.41	*	*	.	-0.45	1.09
Ile	507	.	.	B	B	.	.	.	0.37	0.17	*	*	.	-0.15	1.29
Gly	508	.	.	B	.	.	T	.	0.63	0.36	*	*	F	0.25	0.68
Lys	509	.	.	B	.	.	T	.	0.67	0.10	*	.	F	0.59	0.43
Asn	510	.	.	B	.	.	T	.	0.96	-0.30	*	.	F	1.68	1.02
Leu	511	.	.	B	.	.	T	.	0.61	-0.99	*	.	F	2.32	2.05
Ala	512	.	.	B	.	.	.	.	1.50	-0.99	*	.	F	2.46	1.02
Asp	513	.	.	.	.	T	T	.	1.84	-0.59	*	.	F	3.40	1.02
Lys	514	.	.	.	.	T	T	.	1.49	-0.59	*	.	F	3.06	2.13
Gly	515	.	.	.	.	T	T	.	0.90	-0.79	*	.	F	2.72	3.05
Asn	516	A	.	.	.	.	T	.	1.12	-0.79	.	.	F	1.98	1.84
Gln	517	A	A	.	.	.	.	.	0.82	-0.29	.	*	F	0.79	0.93
Thr	518	.	A	B	.	.	.	.	0.93	0.40	.	*	F	-0.15	0.66
Ala	519	.	A	B	.	.	.	.	0.64	-0.03	.	*	.	0.30	0.80

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Ala	520	.	A	B	.	.	.	.	0.74	0.33	*	*	.	-0.30	0.73
Ile	521	.	A	B	.	.	.	.	0.86	0.69	*	.	.	-0.60	0.79
Arg	522	.	.	B	.	.	.	.	0.86	0.20	*	.	.	0.05	1.53
Tyr	523	.	.	B	.	.	.	.	0.58	-0.30	*	.	.	0.65	2.62
Tyr	524	.	A	B	.	.	.	.	0.31	-0.30	*	*	.	0.45	3.78
Arg	525	.	A	B	.	.	.	.	1.01	-0.34	*	*	.	0.45	1.43
Glu	526	.	A	B	.	.	.	.	1.09	-0.34	*	*	.	0.45	1.79
Ala	527	.	A	B	.	.	.	.	0.98	-0.41	*	*	.	0.30	0.94
Val	528	.	A	B	.	.	.	.	1.01	-0.77	*	*	.	0.60	0.77
Arg	529	.	A	B	.	.	.	.	1.30	-0.34	*	*	.	0.30	0.69
Leu	530	A	.	.	.	.	.	.	0.94	-0.34	*	*	.	0.65	1.37
Asn	531	.	.	.	.	.	T	C	0.09	-0.09	*	*	.	1.05	2.89
Pro	532	A	.	.	.	.	T	.	0.64	-0.09	*	*	F	1.00	1.09
Lys	533	A	.	.	.	.	T	.	0.91	0.41	*	*	.	-0.05	1.80
Tyr	534	.	.	B	.	.	T	.	0.20	0.23	*	*	.	0.25	1.13
Val	535	.	.	B	.	.	.	.	1.01	0.44	*	.	.	-0.40	0.73
His	536	.	.	B	.	.	.	.	1.01	0.41	*	.	.	-0.40	0.58
Ala	537	.	.	B	.	.	T	.	0.41	0.81	*	.	.	-0.20	0.60
Met	538	.	.	B	.	.	T	.	0.02	0.74	*	.	.	-0.20	0.67
Asn	539	A	.	.	.	.	T	.	0.27	0.53	*	.	.	-0.20	0.48
Asn	540	A	.	.	.	.	T	.	0.23	0.43	*	.	.	-0.20	0.77
Leu	541	A	.	.	.	.	.	.	-0.54	0.61	*	.	.	-0.40	0.55
Gly	542	A	.	.	.	.	.	.	0.09	0.69	*	.	.	-0.40	0.28
Asn	543	A	A	.	.	.	.	.	0.69	0.29	*	*	.	-0.30	0.35
Ile	544	A	A	.	.	.	.	.	0.80	-0.11	*	.	.	0.30	0.73
Leu	545	A	A	.	.	.	.	.	0.80	-0.80	*	*	F	0.90	1.45
Lys	546	A	A	.	.	.	.	.	1.61	-0.83	*	*	F	0.90	1.45
Glu	547	A	A	.	.	.	.	.	1.14	-1.23	.	*	F	0.90	3.57
Arg	548	A	A	.	.	.	.	.	1.14	-1.23	*	*	F	0.90	3.57
Asn	549	A	A	.	.	.	.	.	2.03	-1.51	*	*	F	0.90	3.09
Glu	550	A	A	.	.	.	.	.	2.26	-1.51	.	*	F	0.90	3.09
Leu	551	A	A	.	.	.	.	.	2.21	-1.01	*	*	F	0.90	1.60
Gln	552	A	A	.	.	.	.	.	2.21	-1.01	*	*	F	0.90	1.72
Glu	553	A	A	.	.	.	.	.	1.29	-1.41	*	*	F	0.90	1.72
Ala	554	A	A	.	.	.	.	.	0.48	-0.73	*	.	F	0.90	1.72
Glu	555	A	A	.	.	.	.	.	0.18	-0.73	*	.	F	0.75	0.82
Glu	556	A	A	.	.	.	.	.	0.18	-0.74	*	.	F	0.75	0.63
Leu	557	A	A	.	.	.	.	.	-0.41	-0.06	*	.	.	0.30	0.52
Leu	558	A	A	.	.	.	.	.	-1.27	-0.06	*	*	.	0.30	0.30
Ser	559	A	A	.	.	.	.	.	-0.68	0.59	*	*	.	-0.60	0.13
Leu	560	A	A	.	.	.	.	.	-1.57	0.99	*	*	.	-0.60	0.27
Ala	561	A	A	.	.	.	.	.	-1.57	0.99	*	*	.	-0.60	0.23
Val	562	A	A	.	.	.	.	.	-0.97	0.70	*	*	.	-0.60	0.30
Gln	563	.	A	B	.	.	.	.	-0.16	0.74	*	*	.	-0.60	0.56
Ile	564	.	A	B	.	.	.	.	-0.56	0.06	.	*	.	-0.30	0.92
Gln	565	.	.	B	.	.	T	.	-0.33	0.34	.	*	F	0.40	1.08
Pro	566	.	.	B	.	.	T	.	-0.33	0.20	.	*	F	0.25	0.63
Asp	567	A	.	.	.	.	T	.	-0.07	0.30	.	*	.	0.10	0.91
Phe	568	A	.	.	.	.	T	.	-0.36	0.11	.	*	.	0.10	0.53
Ala	569	A	A	.	.	.	.	.	-0.07	0.63	.	*	.	-0.60	0.36
Ala	570	A	A	.	.	.	.	.	-0.07	0.81	.	*	.	-0.60	0.21
Ala	571	A	A	.	.	.	.	.	-0.67	1.21	*	.	.	-0.60	0.40
Trp	572	A	A	.	.	.	.	.	-1.01	1.11	.	*	.	-0.60	0.32
Met	573	A	A	.	.	.	.	.	-1.20	1.04	.	.	.	-0.60	0.32
Asn	574	A	A	.	B	.	.	.	-1.47	1.23	.	.	.	-0.60	0.22
Leu	575	A	A	.	B	.	.	.	-0.88	1.37	.	.	.	-0.60	0.15
Gly	576	.	A	B	B	.	.	.	-0.29	0.86	.	.	.	-0.60	0.27
Ile	577	.	.	B	B	.	.	.	-0.30	0.64	.	.	.	-0.60	0.27

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Val	578	.	.	B	.	.	T	.	-0.51	0.63	.	*	.	-0.20	0.44
Gln	579	.	.	B	.	.	T	.	-0.47	0.63	*	.	F	-0.05	0.37
Asn	580	.	.	B	.	.	T	.	0.46	0.20	*	.	F	0.40	1.05
Ser	581	.	.	.	.	.	T	C	0.10	-0.49	*	.	F	1.20	2.76
Leu	582	.	A	.	.	.	.	C	0.99	-0.34	*	.	F	0.80	1.38
Lys	583	.	A	.	.	.	.	C	1.26	-0.74	*	.	F	1.10	1.49
Arg	584	A	A	.	.	.	.	.	0.67	-0.64	*	.	.	0.75	1.12
Phe	585	A	A	.	.	.	.	.	0.67	-0.53	*	.	.	0.75	1.37
Glu	586	A	A	.	.	.	.	.	0.97	-1.21	*	.	.	0.75	1.19
Ala	587	A	A	.	.	.	.	.	1.48	-0.81	*	.	.	0.75	1.05
Ala	588	A	A	.	.	.	.	.	1.19	-0.43	*	*	.	0.45	1.63
Glu	589	A	A	.	.	.	.	.	1.19	-0.46	.	*	F	0.60	1.47
Gln	590	A	.	.	.	.	T	.	1.58	-0.46	*	.	F	1.00	2.86
Ser	591	A	.	.	.	.	T	.	0.99	-0.47	*	*	F	1.00	4.08
Tyr	592	A	.	.	.	.	T	.	0.69	-0.47	*	*	F	1.00	2.38
Arg	593	A	.	.	.	.	T	.	1.32	0.21	*	*	F	0.25	0.96
Thr	594	A	A	.	B	.	.	.	1.29	-0.19	*	*	.	0.45	1.44
Ala	595	A	A	.	B	.	.	.	1.40	-0.07	*	*	.	0.45	1.25
Ile	596	A	A	.	B	.	.	.	1.81	-0.83	*	*	.	1.05	1.25
Lys	597	.	A	B	B	.	.	.	2.10	-0.83	*	*	.	1.35	1.69
His	598	.	A	B	.	.	.	.	1.74	-1.31	*	*	F	1.80	3.35
Arg	599	.	A	.	.	T	.	.	1.84	-1.06	*	*	F	2.50	7.50
Arg	600	.	.	.	.	T	.	.	2.43	-1.31	*	*	F	3.00	5.80
Lys	601	.	.	.	.	T	.	.	2.66	-1.31	*	*	F	2.70	7.12
Tyr	602	.	.	B	.	.	T	.	2.37	-1.24	*	.	F	2.20	1.95
Pro	603	.	.	.	.	T	T	.	2.16	-0.49	.	.	F	2.00	1.56
Asp	604	.	.	.	.	T	T	.	2.04	0.27	*	*	.	0.95	1.22
Cys	605	.	.	B	.	.	T	.	1.12	0.67	*	.	.	-0.05	1.25
Tyr	606	.	.	B	.	.	.	.	0.73	0.60	*	.	.	-0.40	0.67
Tyr	607	.	.	B	.	.	.	.	1.09	0.60	*	.	.	-0.40	0.40
Asn	608	.	.	B	.	.	.	.	0.49	0.60	*	.	.	-0.25	1.45
Leu	609	.	.	B	.	.	.	.	0.24	0.71	*	*	.	-0.40	0.76
Gly	610	.	.	B	.	.	.	.	0.32	0.71	*	*	.	-0.40	0.76
Arg	611	.	A	B	.	.	.	.	0.57	0.46	*	*	.	-0.60	0.48
Leu	612	.	A	B	.	.	.	.	-0.00	0.06	*	*	.	-0.30	0.97
Tyr	613	.	A	B	.	.	.	.	-0.00	0.06	*	*	.	-0.30	0.81
Ala	614	.	A	B	.	.	.	.	0.92	0.03	*	*	.	-0.30	0.66
Asp	615	A	A	.	.	.	.	.	1.23	0.03	*	*	.	-0.15	1.57
Leu	616	.	A	B	.	.	.	.	0.27	-0.16	*	*	.	0.45	1.37
Asn	617	.	A	B	.	.	.	.	1.08	-0.27	*	*	.	0.45	1.00
Arg	618	.	A	B	.	.	.	.	0.73	-0.77	*	.	.	0.75	1.00
His	619	A	A	.	.	.	.	.	0.51	-0.27	*	.	.	0.45	1.23
Val	620	A	A	.	.	.	.	.	0.51	-0.27	*	.	.	0.30	0.63
Asp	621	A	A	.	.	.	.	.	0.73	-0.27	*	.	.	0.30	0.52
Ala	622	A	A	.	.	.	.	.	0.44	0.23	*	.	.	-0.30	0.38
Leu	623	A	A	.	.	.	.	.	0.44	0.64	*	.	.	-0.60	0.54
Asn	624	A	A	.	.	.	.	.	0.48	-0.00	*	.	.	0.30	0.64
Ala	625	A	A	.	.	.	.	.	0.74	0.40	*	.	.	-0.15	1.02
Trp	626	A	A	.	.	.	.	.	0.43	0.40	*	.	.	-0.15	1.25
Arg	627	A	A	.	.	.	.	.	0.17	0.20	*	.	.	-0.15	1.12
Asn	628	A	A	.	B	.	.	.	0.17	0.44	.	.	.	-0.60	0.82
Ala	629	A	A	.	B	.	.	.	0.21	0.63	*	.	.	-0.60	0.64
Thr	630	.	A	B	B	.	.	.	0.59	-0.29	.	*	.	0.30	0.66
Val	631	.	A	B	B	.	.	.	0.88	0.14	.	*	.	-0.30	0.63
Leu	632	.	.	B	B	.	.	.	0.73	-0.26	.	.	.	0.45	1.08
Lys	633	.	.	B	B	.	.	.	0.43	-0.26	.	*	F	0.60	1.02
Pro	634	.	.	B	.	.	.	.	0.21	-0.36	*	.	F	0.80	1.85
Glu	635	A	A	.	.	.	.	.	-0.07	-0.31	.	*	F	0.60	1.85
His	636	A	A	.	.	.	.	.	0.50	-0.50	*	*	.	0.60	0.93

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Ser	637	A	A	.	.	.	.	.	1.31	0.41	.	*	.	-0.60	0.63
Leu	638	A	A	.	.	.	.	.	1.27	0.39	.	*	.	-0.30	0.59
Ala	639	A	A	.	.	.	.	.	0.88	0.79	.	.	.	-0.60	0.70
Trp	640	A	A	.	.	.	.	.	-0.01	0.90	.	.	.	-0.60	0.51
Asn	641	A	A	.	.	.	.	.	-0.87	1.20	.	.	.	-0.60	0.44
Asn	642	A	A	.	B	.	.	.	-1.38	1.20	.	.	.	-0.60	0.30
Met	643	.	A	B	B	.	.	.	-1.38	1.39	.	.	.	-0.60	0.24
Ile	644	.	.	B	B	.	.	.	-0.79	1.16	*	.	.	-0.60	0.12
Ile	645	.	.	B	B	.	.	.	-0.50	0.76	*	.	.	-0.60	0.13
Leu	646	.	.	B	B	.	.	.	-0.81	0.76	.	.	.	-0.60	0.21
Leu	647	.	.	B	B	.	.	.	-1.16	0.63	*	.	.	-0.60	0.42
Asp	648	.	.	B	B	.	.	.	-0.56	0.37	.	*	F	-0.15	0.60
Asn	649	.	.	.	.	.	T	C	-0.48	0.09	.	.	F	0.60	1.17
Thr	650	.	.	.	.	.	T	C	-0.18	0.09	*	.	F	0.60	1.17
Gly	651	.	.	.	.	.	T	C	0.63	-0.10	*	.	F	1.05	0.71
Asn	652	.	.	.	.	.	T	C	0.86	0.30	.	.	F	0.45	0.76
Leu	653	A	A	.	.	.	.	.	0.86	0.40	.	.	.	-0.30	0.53
Ala	654	A	A	.	.	.	.	.	0.27	-0.09	.	.	.	0.30	0.93
Gln	655	A	A	.	.	.	.	.	-0.28	-0.01	.	.	.	0.30	0.58
Ala	656	A	A	.	.	.	.	.	-0.28	0.23	.	*	.	-0.30	0.53
Glu	657	A	A	.	.	.	.	.	-0.17	-0.03	.	*	.	0.30	0.52
Ala	658	A	A	.	.	.	.	.	0.64	-0.53	*	*	.	0.60	0.58
Val	659	A	A	.	.	.	.	.	0.64	-0.93	*	*	.	0.60	1.00
Gly	660	A	A	.	.	.	.	.	-0.17	-0.93	*	*	.	0.60	0.58
Arg	661	A	A	.	.	.	.	.	0.42	-0.24	*	*	F	0.45	0.48
Glu	662	A	A	.	.	.	.	.	-0.39	-0.74	*	*	.	0.75	1.11
Ala	663	A	A	.	.	.	.	.	-0.69	-0.70	*	*	.	0.60	0.93
Leu	664	A	A	.	.	.	.	.	-0.04	-0.44	*	*	.	0.30	0.33
Glu	665	A	A	.	.	.	.	.	0.30	-0.01	*	*	.	0.30	0.30
Leu	666	A	A	.	.	.	.	.	0.19	0.39	*	*	.	-0.30	0.47
Ile	667	A	.	.	.	.	T	.	0.16	-0.11	*	.	.	0.70	0.95
Pro	668	A	.	.	.	.	T	.	0.44	-0.30	.	.	F	0.85	0.75
Asn	669	A	.	.	.	.	T	.	0.44	0.09	.	.	F	0.40	1.22
Asp	670	A	.	.	.	.	T	.	-0.16	0.09	.	.	F	0.40	1.43
His	671	A	A	.	.	.	.	.	-0.04	0.01	.	.	F	-0.15	0.92
Ser	672	A	A	.	.	.	.	.	0.54	0.37	.	*	.	-0.30	0.49
Leu	673	.	A	B	.	.	.	.	-0.06	0.36	.	.	.	-0.30	0.40
Met	674	.	A	B	.	.	.	.	-0.64	1.04	.	.	.	-0.60	0.24
Phe	675	A	A	.	.	.	.	.	-0.64	1.04	.	.	.	-0.60	0.18
Ser	676	A	A	.	.	.	.	.	-1.47	1.06	*	.	.	-0.60	0.35
Leu	677	A	A	.	.	.	.	.	-1.98	1.01	*	.	.	-0.60	0.26
Ala	678	A	A	.	.	.	.	.	-1.51	1.09	*	.	.	-0.60	0.25
Asn	679	A	A	.	.	.	.	.	-0.87	0.73	*	.	.	-0.60	0.19
Val	680	A	A	.	.	.	.	.	-0.47	0.34	.	*	.	-0.30	0.45
Leu	681	A	A	.	.	.	.	.	-0.17	0.04	*	.	.	-0.30	0.60
Gly	682	A	.	.	.	.	T	.	0.69	-0.06	*	.	F	0.85	0.64
Lys	683	A	.	.	.	.	T	.	1.03	-0.46	.	*	F	1.00	1.74
Ser	684	A	.	.	.	.	T	.	1.08	-0.34	.	.	F	1.00	3.30
Gln	685	A	.	.	.	.	T	.	1.93	-1.03	.	.	F	1.30	6.67
Lys	686	A	.	.	.	.	.	.	2.44	-1.46	.	.	F	1.10	5.77
Tyr	687	A	.	.	.	.	T	.	2.79	-1.07	.	.	F	1.30	5.77
Lys	688	A	.	.	.	.	T	.	2.16	-1.46	.	.	F	1.30	5.77
Glu	689	A	.	.	.	.	T	.	1.64	-1.36	.	.	F	1.30	2.92
Ser	690	A	.	.	.	.	T	.	0.94	-0.67	.	.	F	1.30	1.54
Glu	691	A	A	.	.	.	.	.	0.09	-0.64	.	.	F	0.75	0.66
Ala	692	A	A	.	.	.	.	.	0.38	0.04	*	.	.	-0.30	0.32
Leu	693	A	A	.	.	.	.	.	-0.26	0.04	*	.	.	-0.30	0.47

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Phe	694	A	A	.	.	.	.	.	-1.14	0.16	*	.	.	-0.30	0.28
Leu	695	A	A	.	.	.	.	.	-0.80	0.84	*	.	.	-0.60	0.19
Lys	696	A	A	.	.	.	.	.	-1.39	0.34	.	.	.	-0.30	0.46
Ala	697	A	A	.	.	.	.	.	-0.80	0.16	*	.	.	-0.30	0.54
Ile	698	A	A	.	.	.	.	.	-0.20	-0.23	*	*	.	0.66	1.05
Lys	699	A	A	.	.	.	.	.	0.50	-0.49	*	*	F	0.87	0.82
Ala	700	A	A	.	.	.	.	.	0.72	-0.09	*	*	F	1.23	1.30
Asn	701	.	.	.	.	.	T	C	0.09	-0.09	*	*	F	2.04	1.87
Pro	702	.	.	.	.	.	T	C	0.38	-0.27	*	*	F	2.10	0.95
Asn	703	.	.	.	.	T	T	.	1.02	0.11	.	*	.	1.49	1.25
Ala	704	A	.	.	.	.	T	.	0.94	0.37	.	*	.	0.88	1.22
Ala	705	.	.	B	.	.	.	.	1.19	0.47	.	.	.	0.17	1.07
Ser	706	.	.	B	.	.	.	.	1.19	0.47	.	*	.	-0.19	0.66
Tyr	707	.	.	B	.	.	.	.	0.59	0.47	.	.	.	-0.25	1.05
His	708	.	.	B	.	.	T	.	-0.00	0.66	.	*	.	-0.20	0.86
Gly	709	.	.	B	.	.	T	.	-0.27	0.66	.	*	.	-0.20	0.65
Asn	710	.	.	B	.	.	T	.	-0.49	0.91	.	*	.	-0.20	0.31
Leu	711	.	.	B	.	.	T	.	-0.43	0.84	.	*	.	-0.20	0.19
Ala	712	.	.	B	B	.	.	.	-0.22	1.10	.	*	.	-0.60	0.29
Val	713	.	.	B	B	.	.	.	-0.08	1.17	.	*	.	-0.60	0.25
Leu	714	.	.	B	B	.	.	.	-0.02	0.77	*	.	.	-0.60	0.59
Tyr	715	.	.	B	B	.	.	.	-0.37	1.00	*	.	.	-0.60	0.62
His	716	.	.	B	.	.	T	.	0.41	0.93	*	.	.	-0.20	0.82
Arg	717	.	.	.	.	T	T	.	0.19	0.79	.	.	.	0.35	1.36
Trp	718	A	.	.	.	.	T	.	1.04	0.79	.	.	.	-0.20	0.71
Gly	719	A	.	.	.	.	T	.	1.04	0.03	.	.	.	0.10	0.88
His	720	A	A	.	.	.	.	.	0.70	0.21	.	.	.	-0.30	0.37
Leu	721	A	A	.	.	.	.	.	0.78	0.71	.	.	.	-0.60	0.35
Asp	722	A	A	.	.	.	.	.	0.71	-0.20	.	.	.	0.30	0.72
Leu	723	A	A	.	.	.	.	.	0.97	-0.63	.	.	.	0.75	1.05
Ala	724	A	A	.	.	.	.	.	1.07	-0.63	*	*	.	0.75	1.74
Lys	725	A	A	.	.	.	.	.	1.10	-0.56	*	*	F	0.90	1.63
Lys	726	A	A	.	.	.	.	.	1.02	-0.56	*	*	F	0.90	3.42
His	727	A	A	.	.	.	.	.	0.72	-0.56	*	*	.	0.75	2.37
Tyr	728	A	A	.	.	.	.	.	0.72	-0.67	*	*	.	0.75	1.59
Glu	729	.	A	B	.	.	.	.	1.31	0.01	*	*	.	-0.30	0.66
Ile	730	.	A	B	.	.	.	.	0.46	0.41	.	*	.	-0.60	0.83
Ser	731	.	A	B	.	.	.	.	0.41	0.60	.	*	.	-0.60	0.44
Leu	732	.	A	B	.	.	.	.	0.23	-0.16	.	*	.	0.30	0.42
Gln	733	.	A	B	.	.	.	.	0.17	0.27	.	*	.	-0.30	0.93
Leu	734	.	A	B	.	.	.	.	-0.42	0.07	.	*	.	-0.15	1.01
Asp	735	.	.	.	.	.	T	C	0.17	0.19	.	*	F	0.60	1.23
Pro	736	.	.	.	.	.	T	C	0.12	-0.11	.	*	F	1.31	0.95
Thr	737	.	.	.	.	.	T	C	0.62	-0.09	.	*	F	1.72	1.14
Ala	738	.	.	B	.	.	T	C	0.67	-0.29	.	*	F	1.83	0.99
Ser	739	.	.	.	.	.	.	C	1.48	-0.29	.	*	F	2.04	1.28
Gly	740	.	.	.	.	.	.	C	1.48	-0.71	.	.	F	2.60	1.54
Thr	741	.	.	.	.	.	.	C	1.44	-0.80	.	.	F	2.34	2.44
Lys	742	.	.	B	.	.	.	.	1.41	-0.54	.	.	F	1.88	2.86
Glu	743	.	.	B	.	.	.	.	1.19	-0.50	.	.	F	1.62	2.86
Asn	744	.	.	B	.	.	T	.	0.68	-0.24	*	.	F	1.26	1.63
Tyr	745	.	.	B	.	.	T	.	1.13	-0.04	*	.	.	0.70	0.67
Gly	746	A	.	.	.	.	T	.	1.56	-0.04	*	.	.	0.70	0.76
Leu	747	A	.	.	.	.	T	.	1.56	-0.04	*	*	.	0.70	0.93
Leu	748	A	A	.	.	.	.	.	0.74	-0.44	*	*	.	0.45	1.18
Arg	749	A	A	.	.	.	.	.	0.74	-0.51	*	*	F	0.75	0.99
Arg	750	A	A	.	.	.	.	.	0.18	-0.94	*	*	F	0.90	2.07
Lys	751	A	A	.	.	.	.	.	-0.08	-0.94	*	*	F	0.90	2.07

Table 7 (continued)

Res	Position	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Leu	752	A	A	.	.	.	.	.	0.73	-1.01	*	*	.	0.75	1.05
Glu	753	A	A	.	.	.	.	.	1.59	-0.61	*	*	.	0.60	0.93
Leu	754	A	A	.	.	.	.	.	1.52	-0.61	*	*	.	0.60	0.93
Met	755	A	A	.	.	.	.	.	0.82	-0.61	*	.	.	0.75	2.24
Gln	756	A	A	.	.	.	.	.	-0.08	-0.80	.	*	.	0.75	1.31
Lys	757	A	A	.	.	.	.	.	0.34	-0.16	.	.	F	0.60	1.18
Lys	758	A	A	.	.	.	.	.	-0.04	-0.41	.	.	.	0.45	1.52
Ala	759	A	A	.	.	.	.	.	0.38	-0.60	.	.	.	0.75	1.12
Val	760	A	A	.	.	.	.	.	0.59	-0.57	.	.	.	0.60	0.72

[1753] It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

[1754] The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference. Further, the hard copy of the sequence listing submitted herewith and the corresponding computer readable form are both incorporated herein by reference in their entireties.